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AWARD NUMBER: W81XWH-10-1-0787

TITLE: Regenerative Medicine and Restoration of Joint Function

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REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2012	Annual	27 September 2011 – 26 September 2012

4. TITLE AND SUBTITLE

Regenerative Medicine and Restoration of Joint Function

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0787

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mount Sinai Hospital
Toronto, Canada M5G 1X5

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Currently amputation, arthrodesis (joint fusion), or joint replacement are used to treat a joint with an intra-articular fracture or destroyed by a combat injury. Generation of personalized, anatomically shaped biological implants formed using techniques of regenerative medicine in conjunction with biodegradable biomaterial structures to restore a damaged articular joint surface to normal tissue structure, form and function is one way to overcome the limitations associated with current treatment methods. The aims of this study are to: 1) identify the parameters that generate anatomically shaped bone substitutes of optimal composition and structure with an articulating profile. 2) to develop a source of human chondrocytes that can generate sufficient amounts of a cartilage layer to cover the bone substitute; and 3) to evaluate the structures formed in a preclinical model. The ongoing studies will further our understanding of the regulation of cell differentiation to chondrocytes and the bone substitute properties required to form a biological joint replacement.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	58	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The complexity of extremity injuries as a consequence of battlefield trauma requires multifaceted reconstructions and has resulted in the need to develop entirely new treatment options to achieve limb salvage and thus full rehabilitation. **The overall aim of this research project is to evaluate, using an animal model, large anatomically shaped biological implants formed using techniques of regenerative medicine in conjunction with biodegradable biomaterial structures to restore a damaged articular joint surface to normal tissue structure, form and function prior to progressing to clinical trials to evaluate the application of this treatment approach to humans.** Our multi-disciplinary team is focusing on generating medial tibial plateau and a large segment of the medial femoral condyle (knee joint) biphasic implants (definitive care of battle injuries). The approach we will develop will result in an implant that can be customized contoured to replace the portion of the knee joint disrupted either by an intra-articular fracture or trauma. Being able to generate personalized implants is a critical feature given that most combat injuries are irregularly shaped.

Using an approach that allows for the formation of living tissues for joint reconstruction offers the advantage of functional tissue integration as well as adaptation to loading conditions during use which should avoid implant failure that can result from the fatigue or wear of synthetic biomaterial. Furthermore once we develop the conditions it will be possible to generate an implant of any contour, making this approach particularly appropriate for individuals who have irregular-shaped defects as a result of a combat injury. Two issues that must be overcome before these large biphasic constructs can be used clinically, particularly in the military setting, are the identification of an accessible human cell source to generate a large quantity of cartilage tissue and the methodology to easily, rapidly and reliably generate custom-made CPP bone substitutes of desired shape. This report will describe our results to date (year 2 since grant funding obtained).

BODY

Statement of Work Tasks for this time period

The following describes the work that has been done towards accomplishing the defined tasks described in the grant proposal.

Aim 1: ***To design and make appropriately shaped porous CPP surface replacements for evaluation in a pre-clinical sheep model (0-36 months)***

As there did not seem to be an optimal bone interfacing material, we developed a biodegradable inorganic polymer (calcium polyphosphate, CPP) that can be formed as a porous component by sintering CPP particles of selected size under controlled conditions. The CPP powders are formed from calcium and phosphate and when sintered to form porous structures using appropriate processing conditions that we have developed in previous studies, they do not incite an adverse reaction as they slowly degrade *in vivo.* The degradation rate is dependent on factors such as impurity levels, degree of crystallinity, crystal size and free surface area (related to percent porosity and particle size). The porous CPP structures can be made to have mechanical properties similar to cortical or cancellous bone making them suitable to use as a bone substitute to form joint replacements. The properties (strength and pore size) of the porous CPP can be modulated for the required need through selection of CPP particle size and sintering conditions and can be made sufficient for weight bearing. Although porosity can be as low as 30%, bone ingrowth still occurs likely because of the microporosity. Thus porous CPP is suitable for facilitating cartilage anchorage to bone as well as bone defect filling in a biological joint replacement. However one potential limitation to using porous CPP is the need to generate the substrate easily, rapidly, and reliably in any desired shape in order to be able to repair/regenerate a joint injured during combat or training. One way to accomplish this would be via solid freeform fabrication.

The goal of the studies in aim 1 is to: *Design and make appropriately shaped porous CPP surface replacements for evaluation in a pre-clinical sheep model.* Thus the focus of our studies is to optimize the solid freeform fabrication (SFF) of large anatomically correct shaped segment of the medial femoral condyle as well as the medial tibial plateau in order to generate biphasic implants for evaluation in a sheep model.

Task 1. Material Optimization for the SFF Process:

We have identified appropriate CPP and PVA compositions and particle sizes to facilitate spreadability of the mixture in the newly developed solid freeform (SFF system. In addition, a photopolymer (Ethoxylated-10 bisphenolAdiacrylate) photopolymer solute) ion has been identified and effectively used for making macro-size conformal channels within the CPP substrates. The sacrificial photopolymer is fully removed from the scaffolds post-processing to prevent any side-effects as a result of adverse biological responses. It was shown that the proposed photopolymer is fully removed by exposure of scaffolds to a predefined heat treatment protocol. Material characterizations conducted via Scanning Electron Microscope (SEM) using

an Energy-Dispersive X-ray Spectroscopy (EDS), X-Ray Diffraction (XRD) and Differential Scanning Calorimetry (DSC)-Thermo Gravimetric Analysis (TGA) suggested that the photopolymer was fully disintegrated and no secondary residuals were produced during the heat treatment process. A variety of structures have been made to assess the role of macro- size conformal channels on mechanical strengths. An in vivo study in rabbits to evaluate these materials are ongoing.

Task 2. Optimization of SFF Rolling Compaction Parameters:

The work is ongoing related to optimizing SFF rolling compaction parameters to control the density of green samples. We have used CPP powders for these studies. Based on our results, we concluded that the roller spinning and linear speeds do not change the green density considerably, whereas the stacking layer thickness may effectively control the green density. It was also noted that the roller speeds can only be changed in a specific range where speeds out of this range may lead in crack formation and non-uniformity in the spread powder within the build compartment of SFF system. The results to date indicate that an increase in stacking layer thickness from 150 to 250 um increases the green density from 39%+-2 to 46%+-2. This increases controllability of the final porosity of CPP implants with direct effect on the mechanical strength of the biomaterial. We are working on determining the final implant density. Based on preliminary data we obtained from Ti powders, the higher green density, the higher porosity and the lower mechanical compression strength. This approach will allow us to make functionally graded implants with varying porosity where we can change the stacking layer thickness during the fabrication to arrive at heterogeneous porosity across the implant if desired.

Task 3. Fabrication of the Large Anatomically Correct Segmental Replacement with Multiple CPP Powder Sizes:

We imaged sheep knees by CT and after multiple iterations have developed shaped medial tibial plateau and femoral condyle parts that we believe are suitable to use for the implantation studies (Figure 1). These shapes can be made by solid freeform fabrication. Work is ongoing to determine shrinkage of these parts after sintering at over 900^{o}C.

Task 4 <u>Fabrication of the Targeted Model with Oriented Porosity</u>

We have studied the role of oriented porosity on the mechanical strength of substrates. The mechanical properties of the 35% porous structures were characterized by uniaxial compression testing for compressive strength determination and diametral compression testing to determine tensile strength. Fracture cleavage surfaces were analyzed using scanning electron microscopy. The effects of the fabrication process on the microarchitecture of the CPP samples were also investigated. Results suggest that the orientation of the stacked layers has a substantial influence on the mechanical behavior of the SFF-made CPP samples. The samples with layers stacked

parallel to the mechanical compressive load are 48% stronger than those with the layers stacked perpendicular to the load. However, the samples with different stacking orientations are not significantly different in tensile strength. The observed anisotropic mechanical properties were analyzed based on the physical microstructural properties of the CPP structures.

Aim 2: ***Optimize the conditions to form cartilage on larger surfaces using human chondrocytes*** **(0-36 months)**

One major problem limiting the clinical application of bioengineered cartilage for joint repair is identifying a source of sufficient numbers of differentiated chondrocytes to form enough articular cartilage to cover a large surface replacement. Chondrocytes de-differentiate when passaged (to expand cell number) even once in monolayer culture. A variety of approaches have been developed to circumvent this but none entirely successful. To translate this biphasic implant approach into clinical practice we must be able to generate large amounts of cartilage from human chondrocytes, which we can do reliably using a co-culture approach. However this requires, at present, the use of bovine chondrocytes. The following experiments are designed to determine the conditions that will eliminate the use of bovine cells in the formation of human cartilage *in vitro* either by using non-committed human induced progenitor (iPS) cells or alternatively by identifying the factor(s) regulating chondrocyte re-differentiation of passaged human cells.

Task 5: To generate chondrocytes from iPS cells that can serve as a source of chondrocytes to form the cartilage layer of the large biphasic implants

5a. Generate iPS cells from chondrocytes and characterize phenotype: We have identified the conditions to form iPS cells from nucleus pulposus cells and applied that methodology to form iPS cells from chondrocytes. The approach entails transfecting cells with the genes c-Myc, Klf4, Oct4 and Sox2 (single piggyBac (PB) cassette whose expression is under doxycycline regulation and will also provide neomycin resistance). The transfected cells are selected for by monolayer culture in the presence of G418 until 100% of the untransfected (control) cells have died. The G418 is then removed and the transfected cells are allowed to proliferate. We evaluated a number of experimental conditions to generate these cells. We have tried different ways to transfect chondrocytes including fugene, lipofectamine, and Neon with transfection rates of less than 10%. A number of pretreatments (e.g. enzymatic) have been attempted as well as varying cell density during transfection. Chondrocytes after different numbers of passage or even freshly isolated from cartilage have been tried as have both bovine, sheep and human chondrocytes. To date we have not been able to generate colonies, indicative of transdifferentiation to embryonic cells (iPS). Under one condition, transfection of constitutively active genes, have we been able to generate a colony but this is not useful for use clinically as they will not be amenable to differentiation back to chondrocytes.

As an alternate approach, iPS cells were derived from human fibroblasts. Within the last year, we have generated induced pluripotent stem cells (iPSCs) from human fibroblasts. iPSCs were

generated by retroviral infection of Oct4, Sox2, Klf4, and c-Myc in normal fibroblasts. Over 20 clones were generated. Two iPSC clones were fully characterized. These cells maintain normal karyotype after reprogramming. They express the pluripotency markers SSEA-4 and TRA-1-60. Using an *in vitro* embryoid body (EB) outgrowth differentiation assay, all iPSC lines were shown to differentiate to all three germ layers because they expressed smooth muscle actin (mesoderm marker), GATA4 (endoderm marker), and βIII-tubulin (ectoderm marker). We are now in the process of determining the method to induce chondrogenesis of these cells. Our approach will start with using methods developed to generate chondrocytes from mouse embryonic cells as well as our established co-culture methods.

Task 6: To develop use of human chondrocytes for cartilage tissue formation by identifying putative differentiation factor(s)

6a. Determine if young human chondrocytes induce redifferentiation of passaged human chondrocytes:

Passaging of chondrocytes in culture results in dedifferentiation of the cells and loss of their ability to form hyaline (articular) cartilage tissue. Co-culturing human passaged chondrocytes with small numbers of primary bovine chondrocytes (bP0hP2) induced redifferentiation of human chondrocytes. Primary adult human chondrocytes (AhP0), either alone or in co-culture (to induce redifferentiation of passaged cells), did not result in cartilage tissue formation. As bovine articular chondrocytes are harvested from skeletally immature animals, we examined if the inability of adult human chondrocytes to form cartilage tissue is dependent on age or species differences between human and cows. Primary and passaged human fetal chondrocytes were cultured alone and in co-culture. Primary human fetal chondrocytes formed cartilage tissue whereas passaged human fetal chondrocytes did not form cartilage tissue rich in proteoglycans when compared to primary bovine chondrocytes as evidenced by toluidine blue staining and biochemical analysis (Figure 2). Additionally, human fetal primary chondrocytes when combined in co-culture with bovine (fhP0bP2) or fetal human passaged cells (fhP0fhP2) were not able to form hyaline cartilage tissue as determined histologically and biochemically. However bovine chondrocytes induced passaged human chondrocytes to form cartilage tissue. (Figure 3) Of note, levels of proteoglycans in tissues formed by culture expanded chondrocytes grown in co-culture with fetal primary chondrocytes were significantly greater than when fetal primary cells were cultured alone. This indicates that primary human fetal chondrocytes do have some capacity to induce redifferentiation, but not to the extent of primary bovine chondrocytes. The data suggests that human cells do not have the same capacity as bovine chondrocytes to induce redifferentiation.

6b. Identify factor(s) present in conditioned media of co-cultures using mass spectroscopy:

We have concentrated on identifying the conditions that will allow us to generate cartilage from human chondrocytes. Tissue formed in the presence of fetal bovine serum can not be easily used in humans because of the incorporation of foreign (xenogeneic) antigens. Our studies using

bovine chondrocytes have demonstrated that it is possible to generate cartilage tissue under serum-free conditions. We have characterized chondrogenic gene changes in the differentiating cells to identify the optimal time to evaluate the conditioned media by mass spectroscopy. We observed that gene expression levels of Sox9 (a transcription factor involved in chondrogenesis and expressed by chondrocytes) peaked after the first week of culture, similarly levels of aggrecan, and type II collagen increased throughout the first week, while levels of type I collagen decreased significantly after the first week. By 8 days of culture, sox 9 levels were similar to differentiated chondrocytes and the levels of type I collagen had decreased significantly and type II collagen has increased significantly when compared to expression levels at day 0 (dedifferentiated chondrocytes). This suggested that our analyses should be performed on conditioned media from the first week of culture.

To characterize the conditioned media we have done mass spectroscopy studies to identify marker(s) indicative of cells that are undergoing redifferentiation and the factor(s) responsible for this redifferentiation process. The samples are alkylated with iodoacetamide and desalted using NAP5 columns. The samples are trypsin-digested and the resulting peptides are separated by cation-exchange liquid chromatography. The peptides are subjected to mass spectrometry identification with LC-MS/MS, on the ThermoFisher OrbiTrap XL Mass Spectrometer. The raw spectra are analyzed using Mascot and X! Tandem software.

Mass spectrometry identified 1845 total proteins in the combined secretomes of the bP0 and bP2 cultures. After filtering each groups proteins for confidence (95%) using Mascot, 843 proteins remained in bP0 secretome and 1140 remained in the bP2 secretome. Protein overlap between groups was 50.6%, while 36% of proteins were found only in the bP2 cultures and 13.4% found only in the bP0 cultures. Peptide overlap was 43.4%, with 43.1% of peptides detected only in bP2 cultures and 13.5% of peptides detected only in bP0 cultures (Figure 4). After comparing the proteins found in each group, 171 proteins were found at significantly different levels as determined by students t-test while having a spectral count difference of at least 10. Analysis of the proteins present in the secretome of these two cells types revealed many differences in types and amounts of proteins secreted by these cells. Collagens type I, II, and XII, were all found at significantly different levels in the secretomes of bP2 and bP0 cells. Chondrocyte specific proteins such as collagen type II were found at high levels in the media conditioned by bP0 cells, while collagen type I, indicative of dedifferentiated chondrocytes, was present at higher levels in bP2 conditioned media. One growth factor, connective tissue growth factor, and proteins that can modulate growth factor functionality, such as insulin-like growth factor-binding protein 2, were detected. Our analyses are still ongoing. This work will form the basis of an abstract to be submitted to Osteoarthritis Research Society in December.

Aim 3: ***Pre-clinical evaluation of the joint replacement in vivo (months 18-36)***

The goal of these experiments are to evaluate whether a partial hemi-joint replacement composed of a medial tibial plateau and a large, anatomically correct biphasic segmental replacement of the femoral condyle generated *in vitro* using SFF can be used to repair large joint surfaces in a pre-clinical (sheep) model. As the bone substitute can be shaped to model any part of a joint surface,

the parts utilized in these studies will serve as proof-of-concept of the effectiveness of our approach and will be the first step towards the ultimate goal of implanting a total large joint replacement. Furthermore, as it is a large animal clinically relevant sized implants are being evaluated.

Task 7: Pre-clinical evaluation of the joint replacement in vivo:

We have developed the techniques to image sheep knees by CT and we have developed shaped medial tibial plateau and femoral condyle parts as described in Aim 1 that we believe are suitable to use for the implantation studies (Figure 1).

We have received approval to do the study from the local animal care committee and have submitted paperwork for approval of the animal study to DoD. We are awaiting approval to proceed. This aim is dependant on the outcome of aims 2 and 3 and is thus the least advanced as would be expected.

KEY RESEARCH ACCOMPLISHMENTS

1) identified appropriate CPP and PVA compositions, CPP particle sizes, and photopolymer to facilitate solid freeform fabrication system (additive manufacturing) to generate parts fabricated from CPP

2) demonstrated that orientation of the stacked layers appears to have a substantial influence on the mechanical behavior of the solid freeform fabrication made CPP samples

3) generated calcium polyphosphate biomaterial with oriented porosity

4) animal studies evaluating calcium polyphosphate bone substitutes with oriented porosity formed by solid freeform fabrication in vivo is in progress

5) design and formation of a large anatomically correct segmental replacement part by solid freeform fabrication methodology for medial tibial plateau and femoral condyle

6) determined that chondrocytes do not easily transdifferentiate into iPS cells and that fibroblasts may be a better source of cells

7) determined that it is not the age of the human chondrocytes that influence the ability of cells to induce redifferentiation of passaged human cells.

8) serum-free culture conditions have been developed to induce chondrocytes to form cartilage tissue and the tissue characterized

9) analysis of secretome of chondrocytes forming cartilage tissue by mass spectroscopy and have identified matrix molecules present predominately in redifferentiating chondrocytes

10) mass spectroscopy has identified a growth factor present in the secretome (conditioned media) of redifferentiating cells

REPORTABLE OUTCOMES

1) calcium polyphosphate can be used to generate a hemi-tibial plateau and femoral condyle segment by solid freeform fabrication

2) the strength of the porous biomaterial can be modulated by the orientation of layering of the calcium polyphosphate

3) generated functionally graded implants with varying porosity where we can change the stacking layer thickness during the fabrication to arrive at heterogeneous porosity across the implant.

4) developed shaped medial tibial plateau and femoral condyle segmental parts suitable to use for joint repair

5) passaged bovine chondrocytes can be induced to form cartilage tissue under serum-free conditions

6) bovine but not human chondrocytes secrete a factor(s) that induces passaged human chondrocyte redifferentiation

7) mass spectroscopic analysis has confirmed the secretion of matrix molecules and specific growth factors under conditions of redifferentiation

8) submission of manuscript to International Journal of Advanced Manufacturing Technology (see appendices)

9) abstracts accepted (Biomedical Engineering Society (BMES)) for presentation or being written for submission to national meetings describing results to date

CONCLUSIONS

The data collected to date suggests that we can form custom-made calcium polyphosphate bone substitutes of desired shape using solid freeform fabrication. The ongoing studies will result in the development of a methodology to form cartilage by human chondrocytes. The next steps, once the aim 2 is completed. will be to implant the constructs in the large animal model.

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PUBLICATIONS submitted

Y. Shanjani, E. Hu, R. M. Pilliar, and Ehsan Toyserkani, "Influence of Layer Stacking Orientation on Mechanical Characteristics of Solid-Freeform-Fabricated Porous Calcium Polyphosphate Structures" *Proc. of the Canadian Biomaterials Symposium*, pp: 237-238, Vancouver, BC, Canada, 2011.

M. Vlasea, Y. Shanjani A. Bothe, R. Kandel, and E. Toyserkani, "A novel additive manufacturing technique for realization of bio-ceramic structures with micro-scale channels", *Submitted to the International Journal of Advanced Manufacturing Technology*, 2012.

M. Vlasea, Y. Shanjani, E. Toyserkani, R. Kandel, "Characterization of Calcium Polyphosphate Scaffolds with Embedded Micro-Channels for Osteochondral Tissue Replacement or Augmentation" *Proc. of 2012 Annual Meeting of Biomedical Engineering Society*, October 24-27, Atlanta, USA,

APPENDICES

A Novel Additive Manufacturing Technique for Realization of Bio-Ceramic Structures with Micro-Scale Channels

--Manuscript Draft--

Manuscript Number:	IJAMT-D-12-09737
Full Title:	A Novel Additive Manufacturing Technique for Realization of Bio-Ceramic Structures with Micro-Scale Channels
Article Type:	Original Research
Keywords:	Additive manufacturing, Rapid prototyping, Layered manufacturing Micro-syringe deposition, Calcium polyphosphate, Sacrificial photopolymer, Bio-ceramic
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Abstract:	This article presents a novel rapid layered manufacturing approach based on a combined Additive Manufacturing (AM) process and a UV-based Micro-Syringe Deposition (μSD) technique to be used in the fabrication of bio-ceramic structures with controlled micro-sized channels for bone and osteochondral tissue regeneration. In the proposed rapid manufacturing method, micro-scale sacrificial photopolymer networks are integrated within the manufactured part by depositing the photopolymer on selected bio-ceramic powder layers using an injection system. This AM-μSD method along with a post-processing protocol can potentially overcome current limitations of traditional powder-based AM approaches that are restricted in terms of complexity of internal architecture and feature size. However, the material system, including sacrificial photopolymer and bio-ceramic, and the post-processing protocol must carefully be selected to ensure that the final structures are free from harmful residuals preventing any detrimental side-effects in post implantation. In this study, calcium polyphosphate (CPP) was used as the substrate material, polyvinyl alcohol (PVA) solution as the binding agent and ethoxylated (10 bisphenolA diacrylate) (EBA) photopolymer solution as the sacrificial element. Material characterization suggests that the proposed material system along with the heat treatment protocol is suitable for the targeted applications where micro-scale channels within the implant are produced by AM-μSD.

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April 15, 2012

The International Journal of Advanced Manufacturing Technology
Professor David W. Russell
Penn State Great Valley,
30 East Swedesford Road, Malvern, PA 19355, USA

Dear Professor Russell:

I would like to submit the manuscript of our paper entitled "A Novel Additive Manufacturing Technique for Realization of Bio-Ceramic Structures with Micro-Scale Channels" to be considered for publication in The International Journal of Advanced Manufacturing Technology. This manuscript has not been published previously and is not under review for publication in any other journal or proceeding. If this manuscript is accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written permission of the publisher.

Sincerely,

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A Novel Additive Manufacturing Technique for Realization of Bio-Ceramic Structures with Micro-Scale Channels

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Submitted Electronically to: Journal of Advanced Manufacturing Technology

Submission Date: April 15, 2012

Number of Pages: **22**

Number of Figures: **8**

Number of Tables: **1**

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A Novel Additive Manufacturing Technique for Realization of Bio-Ceramic Structures with Micro-Scale Channels

Abstract

This article presents a novel rapid layered manufacturing approach based on a combined Additive Manufacturing (AM) process and a UV-based Micro-Syringe Deposition (μSD) technique to be used in the fabrication of bio-ceramic structures with controlled micro-sized channels for bone and osteochondral tissue regeneration. In the proposed rapid manufacturing method, micro-scale sacrificial photopolymer networks are integrated within the manufactured part by depositing the photopolymer on selected bio-ceramic powder layers using an injection system. This AM-μSD method along with a post-processing protocol can potentially overcome current limitations of traditional powder-based AM approaches that are restricted in terms of complexity of internal architecture and feature size. However, the material system, including sacrificial photopolymer and bio-ceramic, and the post-processing protocol must carefully be selected to ensure that the final structures are free from harmful residuals preventing any detrimental side-effects in post implantation. In this study, calcium polyphosphate (CPP) was used as the substrate material, polyvinyl alcohol (PVA) solution as the binding agent and ethoxylated (10 bisphenolA diacrylate) (EBA) photopolymer solution as the sacrificial element. Material characterization suggests that the proposed material system along with the heat treatment protocol is suitable for the targeted applications where micro-scale channels within the implant are produced by AM-μSD.

Keywords: Additive manufacturing, Micro-syringe deposition, Calcium polyphosphate, Sacrificial photopolymer, Bio-ceramic

1. Introduction

Musculoskeletal diseases involve a variety of conditions such as arthritis, osteoporosis, traumatic musculoskeletal injuries, spinal injuries and spinal deformities [1]. Although they are mostly non-life-threatening, these conditions can become debilitating, diminishing the quality of life of the affected individual by causing ongoing pain, discomfort, inflammation and restrictions in range of motion [1]. Aside from clinical repercussions, musculoskeletal conditions represent a major financial burden on the healthcare sector, as the current treatment for advanced joint trauma is to fully or partially replace the damaged joint using tissue grafts or artificial prosthetics to restore near-normal functions of the articulating surfaces [1-2]. There is a pressing need for a more successful approach to joint replacement and joint reconstruction procedures. One such approach focuses on tissue engineered biocompatible and bioresorbable implants that can replace the damaged area of the joint, gradually mature and seamlessly integrate with the native tissue [3]. The idea is to manufacture an implant that temporarily provides mechanical support and acts as a template for new bone tissue formation. The design of such an implant has to consider the internal architecture of bone, which entails interconnected porosity as well as complex networks of channels. Figure 1 illustrates features of natural bone converted into an ideal network of micro-size channels and interconnected pores that could be integrated throughout the volume of the implant.

The desired range of interconnected pore or channel size varies between 100-500 μm [4-6]. The organized networks of micro-channels facilitate cell attachment, encourage bone in-growth, promote metabolic exchange and implant fixation [7-8]. The channels are also used to mimic the natural bone porous macro architecture evolved as an adaptation to mechanical loadings [9].

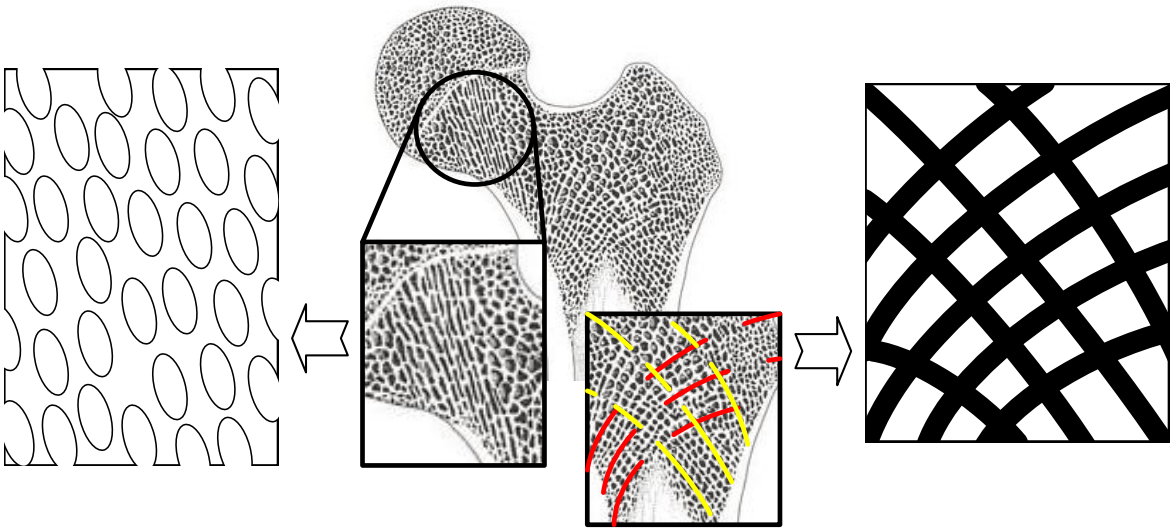

Figure 1 Natural bone morphology and a potential network of channels and/or macro-pores that can be integrated in the implant structure to mimic natural bone architecture

Powder-based additive manufacturing (AM) approaches via three-dimensional printing show promise in the field of tissue engineering and more specifically for bone and osteochondral implant fabrication [4, 10]. The powder-based AM approaches, however, are currently restricted in terms of size of implants with interconnected channel features that can be produced. For larger implants with complex internal features, it is very difficult to remove trapped residual material from inside the implant structure, therefore porosity and internal channel configuration cannot be properly controlled [10]. The current capabilities of powder-based AM techniques cannot reliably allow for features below 500 μm in size and complex channels [10]. These limitations need to be surpassed in order to produce large scale anatomically-shaped implants with controlled densities, micro- and macro-porosities and interconnected networks. The use of AM in the fabrication of bio-ceramic structures with heterogeneous internal properties such as micro-scale conformal channels calls for the development of improved fabrication platforms [10].

The aforementioned implant fabrication limitations can be overcome by combining the capabilities of powder-based AM techniques with a micro-Syringe Deposition (μSD) process. Using such a collaborative processes, a sacrificial element can be injected within the powder layer corresponding to the part slice being manufactured using a computer aided design tool and a manufacturing platform. This manufacturing process requires a post-processing heat treatment procedure used to concomitantly sinter the structures and to remove the sacrificial polymeric element in order to eventually form micro-scale networks of channels within the part. This proposed novel fabrication method can be considered to be one of the more appropriate techniques for fabricating ceramic structures where a refined feature size and internal part complexity are important criteria.

An important step toward manufacturing of bone and osteochondral implants is to identify the biomaterials required: the bio-ceramic and the appropriate polymer that is not only injectable via a micro syringe system, but also a material which leaves no harmful residuals after the post-heat treatment process. For bone and osteochondral tissue engineering applications, the selected ceramic should exhibit excellent biocompatibility and biodegradability while being osteoconductive, thus allowing rapid bone ingrowth into the implant. The fabricated porous implants produced using the selected material should also have appropriate load bearing properties. Also, the selected photopolymer should be curable in a short period of time and the cured photopolymer should be mechanically strong to guarantee the integrity of the deposited material on the ceramic substrate during the manufacturing process.

This work investigates the feasibility of producing bio-ceramic structures with micro-channels using the proposed novel AM-μSD technique. The main purpose of this study is to demonstrate that the proposed biomaterial system along with the heat treatment protocol is

suitable for the suggested AM-μSD process and that the photopolymer is fully removed during the implant post-processing stage to prevent host immune responses or other detrimental side-effects post implantation.

2. Materials and Methods

2.1. Structure Material

A new type of ceramic called calcium polyphosphate (CPP) is amongst one of the more promising materials for producing structures for bone replacement. Calcium polyphosphate is a form of condensed calcium phosphate with a lower ratio of Ca:P that forms linear polymeric-like phosphate chains [11]. This material is biocompatible and biodegradable, forming calcium orthophosphate, which is a “naturally occurring and readily metabolizable substance"[11]. The material is also osteoconductive, allowing rapid bone ingrowth into the implant [12] as suggested by *in vitro* [11] and *in vivo* [13] studies. Recent investigations show that depending on the CPP powder particle size, the pore interconnectivity was moderate and that the compression strength shows promise for bone tissue engineering [11, 14].

In this study, calcium polyphosphate (CPP) powder with batch sizes of 40-45μm and 75-100μm was used as the implant material along with polyvinyl alcohol (PVA) 4wt% solution in de-ionized water (DI) as a binder. The binder was delivered by a peizo-based print-head (XAAR, 1001 model, Xaar, Cambridge, UK).

2.2. Photopolymer Material and Preparation

The proposed photopolymer solution is comprised of a monomer, a reactive dilutant mixed to achieve the desired injectability and a photoinitiator (PI) to control the UV light sensitivity. Ethoxylated (10 bisphenolAdiacrylate) (EBA) (Ebecryl 150, Cytec, New Jersey,

USA) was used as a monomer. Cellulose acetate butyrate (CAB) (Sigma Aldrich, Oakville, Canada) was dissolved in acetone and functions as dilutant. Phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (Irgacure 819) (Sigma Aldrich, Oakville, Canada) was utilized as a PI. The components were utilized as received and mixed in solution at room temperature with a composition of 13wt% CAB, 56wt% EBA, 30.5wt% acetone, and 0.5% PI. A PI concentration of 0.5wt% resulted in an acceptable photopolymerization time of approximately 3 s when exposed to a UV light (RX FireFly 25x10AC395, Phoseon Technology, Hillsboro, US) radiated from a maximum distance of 10 cm. For the sake of simplicity, in this study, the solution is referred to as EBA*.

2.3. Fabrication Process

2.3.1. Additive Manufacturing Process

The initial stage of the AM-μSD process is to design a three dimensional computer-aided design (CAD) model based on the required bio-structures while considering the capabilities of the system and the morphological properties of the natural bone section to be replaced. The second stage in the AM-μSD process is the actual fabrication of the implant. The combined AM-μSD technique employs CPP ceramic powder as the substrate material. The structure is built in a layer-by-layer fashion in multiple steps as shown in Figure 2. Within each layer, the powder is first spread using a counter-rotating roller mechanism and then bonded together at specific locations by injecting a binder using an inkjet-like printing technology. A new layer of powder is subsequently spread on top. The cycle is repeated until the part is completed. On specific layers, the photopolymer is injected using a micro syringe mechanism. The novel AM-μSD system for such a process has been developed in-house. Figure 3 depicts a view of this system.

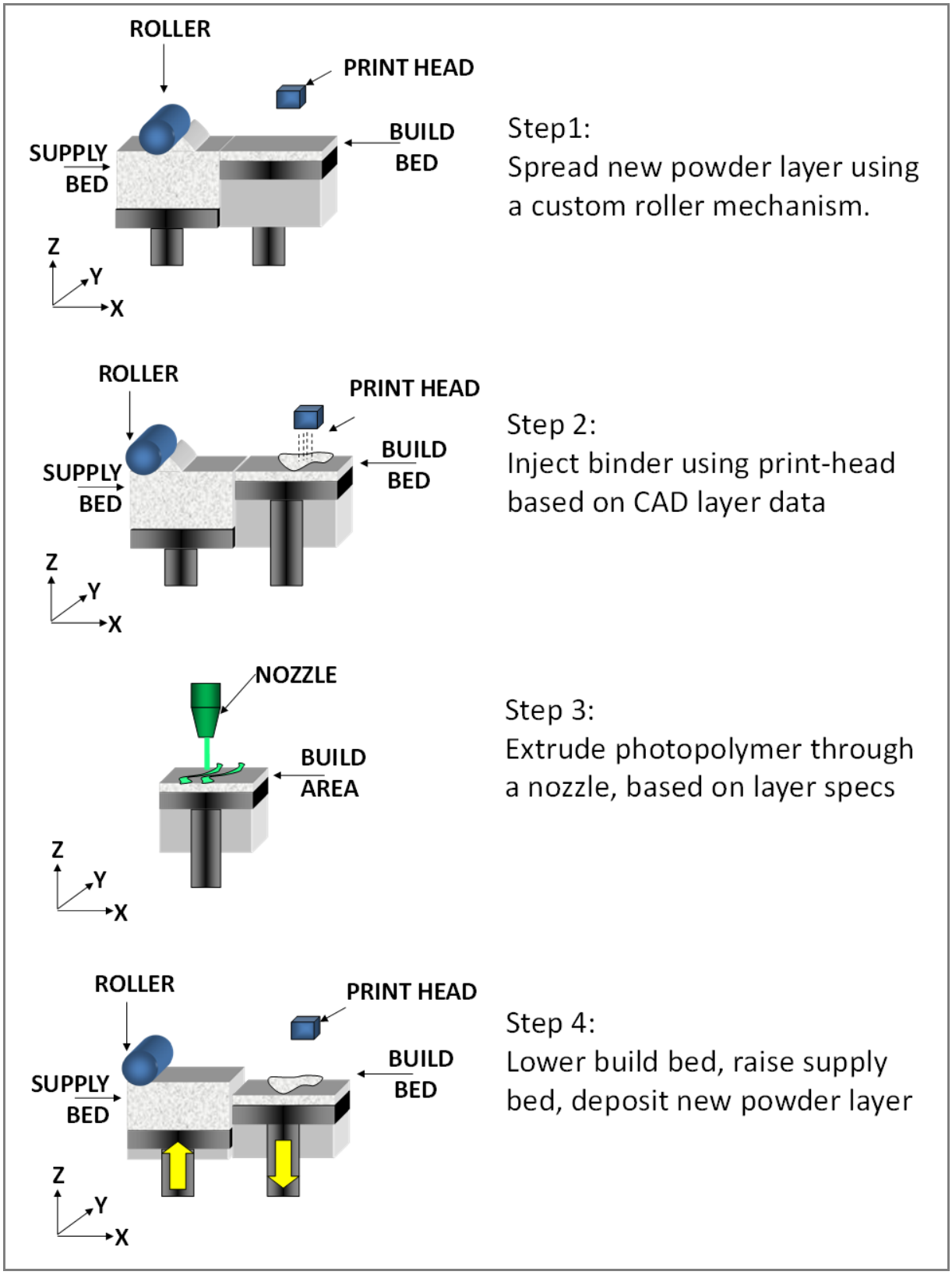

Figure 2 Schematic of the novel AM-μSD fabrication process for production of entire implants in a layer-by-layer fashion through sequential Steps 1 to 4

2.3.2. Sacrificial Photopolymer Deposition Process

The photopolymer was injected using a custom µSD system under an intermittent UV-light (Phoseon Technology, FireFly 25x10, Oregon, USA) exposure from a distance of 10 cm. Different injection process parameters were tested which will be discussed in a separated article. The injection head velocity varied from 1 to 2.5 mm/s and the value of the flow rate was between 2.5 to 10 µL/min. A nozzle size of 150 µm was used. The proposed photopolymer achieved consistent and accurate deposition tracks proving that this photopolymer was a feasible choice in terms of injectability and curing time. Figure 3c shows a typical network of photopolymer on a powder substrate.

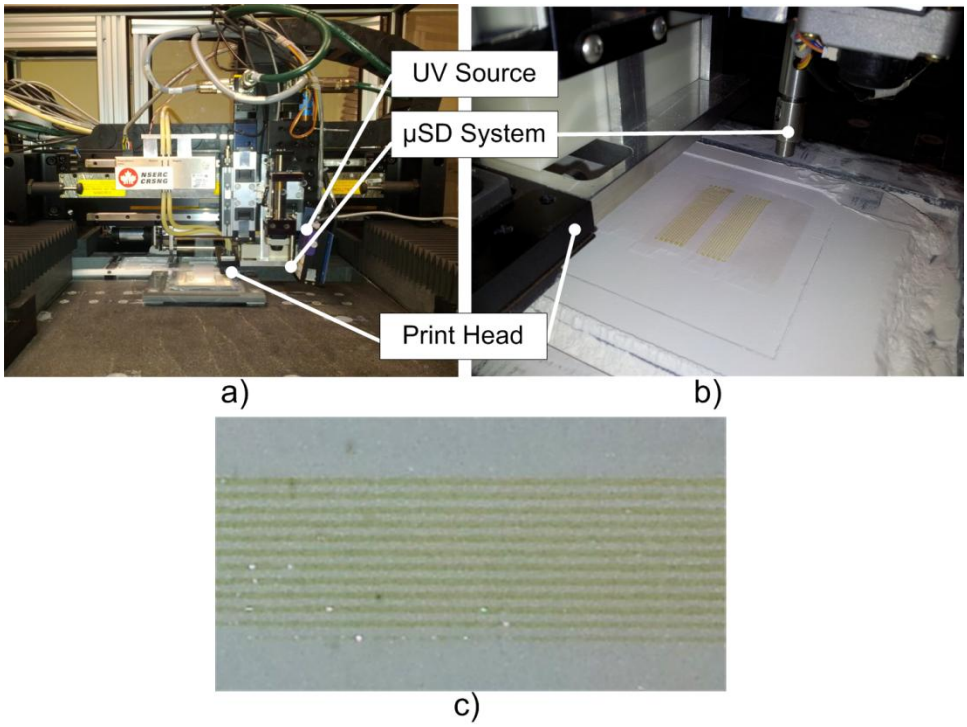

Figure 3 a) and b) views of in-house developed AM-µSD fabrication system, c) close-up view of a typical manufactured layer presenting the deposited photopolymer structure on the powder substrate

2.3.3. Post-process - Binder Removal, Photopolymer Removal and Sintering

The completed parts were left overnight and exposed to a heat treatment to fully cure or anneal the green part. Samples were air-annealed in a high-temperature furnace (EQ-GSL-1500X-40, MTI Corporation, Richmond, US) with an established heat treatment protocol

[15]. The heating rate was 10 ˚C/min from room temperature to 400 ˚C, holding for 1hr followed by another heating cycle of 10 ˚C/min up to 600 ˚C and a dwell time of 1 hr, ending with another heating cycle of 10 ˚C/min up to 950 ˚C with a dwell time of 1 hr to ensure the formation of crystalline CPP and vaporization of any residual carbon from the structures. The cooling process was controlled for four hours.

2.4. Sample Preparation and Characterizations

Samples were manufactured using the methodology explained before. To this end, 25 layers (100 μm thick, 50×50 mm^2 area) of CPP was first spread and bonded. Subsequently, the μSD system was used to deposit four tracks of photopolymer (Sample A, 4.5 mm long, 2 mm apart) for optical microscopy and (Sample B, 20 mm long, 2 mm apart) intended for scanning electron microscopy. The tracks were covered with another ten layers of CPP as previously described. The part was sintered using the established post-process heat treatment protocol.

2.4.1. Geometrical Properties of Formed Channels

To ensure the formation of channels after the heat treatment protocol, a sample (Sample A) with integrated channels was embedded in epoxy, dissected in vertical plane using a band saw and polished to observe the formation of channels under an optical microscope (Olympus Japan BH Optical Microscope, Carsen Medical & Scientific CO. LTD., Markham, Ontario, Canada). In addition, to better observe the quality of the formed channels after the heat treatment protocol, another sample (Sample B) with integrated channels was dissected in the longitudinal plane and observed using a scanning electron microscope (SEM, JSM-6460, Jeol, Akishima, Tokyo) at 20 kV accelerating voltage. To

make the sample electrically conductive, they were sputter-coated with a 10 nm gold layer (Desk II, Denton Vacuum, LCC, Moorestown, US).

2.4.2. Polymer Thermal Decomposition Properties

One of the initial steps in validating the AM-μSD methodology was to establish the thermal degradation pathway and degradation temperature profile of the photopolymer using a Differential Scanning Calorimetry (DSC)-Thermo Gravimetric Analysis (TGA) (SDT Q600, TA Instruments, Grimsby, Canada) under the same heat-treating protocol used for curing the samples. The EBA* photopolymer solution was injected through our micro-syringe and UV cured to form a small drop weighing 10 mg. The same experimental analysis was performed on 10 mg of pure PVA polymer to ensure that this polymer also fully degrades during the heat-treating protocol.

2.4.2.1. Surface Chemical Composition

For investigating the surface chemical composition of CPP structures after sintering, two types of heat-treated samples were prepared: a CPP-PVA baseline implant and a CPP-PVA-EBA* implant containing the photopolymer. The chemical composition after sintering of the CPP-PVA and CPP-PVA-EBA* samples was analyzed using a Field Emission Scanning Electron Microscope (FESEM) with Energy-Dispersive X-ray Spectroscopy (EDAX) (LEO1530, Carl Zeiss AG., Peabody, US). Due to non-conductivity of samples, 10 nm gold layer coating was deposited using a sputter coater (Desk II, Denton Vacuum, LCC, Moorestown, US) prior to FESEM-EDAX analysis. Both samples were investigated at a magnification of 350X. Two investigation areas were the focus of each sample, 250 μm^2 and 50 μm^2.

2.4.2.2. Surface Material Composition

X-Ray Diffraction (XRD) was used to examine the material composition and crystallinity of the heat-treated CPP-PVA and CPP-PVA-EBA* samples to establish if there are any significant differences introduced through the presence of the photopolymer during the fabrication process. The XRD analysis equipment (RAPID II X-ray Detector, Rigaku, Texas, US) operated with 50 kV and 40 mA. The exposure time was 15 seconds and the angle of interest was selected between 15˚-65˚.

3. Results

3.1. Geometrical Properties of Formed Channels

As shown in Figure 4, the combined AM-μSD process resulted in the creation of channels inside CPP porous implants. The resulting channels have a post-sintering shape with a width of ~450 μm and height of ~250 μm. The channels were originally spaced 2 mm apart, however, due to the part shrinkage during the post-processing heat treatment, the distance between channels was reduced by ~12.5%.

The SEM imaging summarized in Figure 5 shows that qualitatively, the tracks produced using the AM-μSD method are visible, well defined and consistent. Some channels show variability in terms of channel width (Figure 5a) from approximately 275 μm to 225 μm, while the channel in Figure 5b is relatively constant in width, around 260 μm. Similarly to the sample shown in Figure 4, the spacing between channels is reduced by ~12.5% due to the part shrinkage during the post-processing heat treatment.

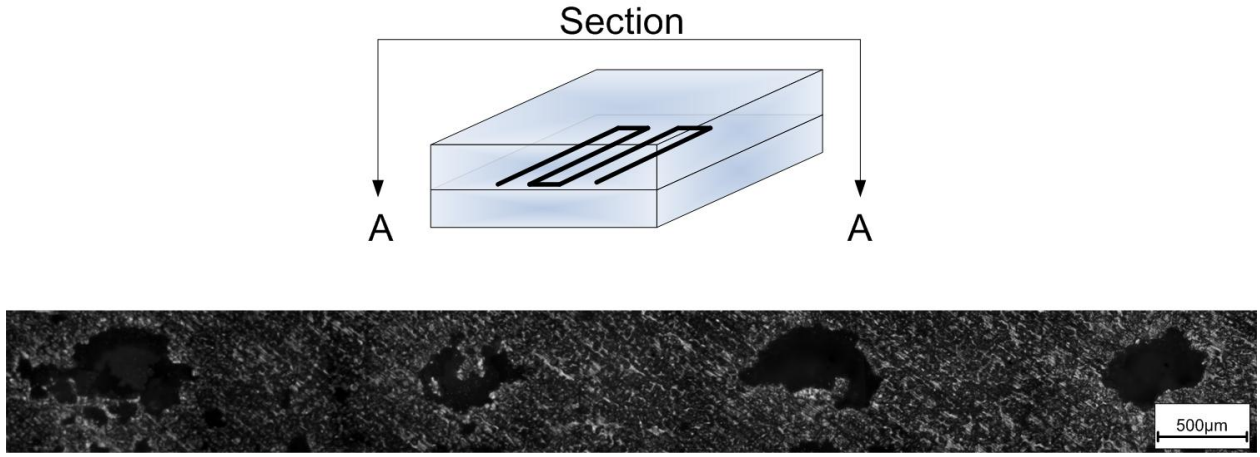

Figure 4 Cross section of CPP part with integrated channels after heat treatment

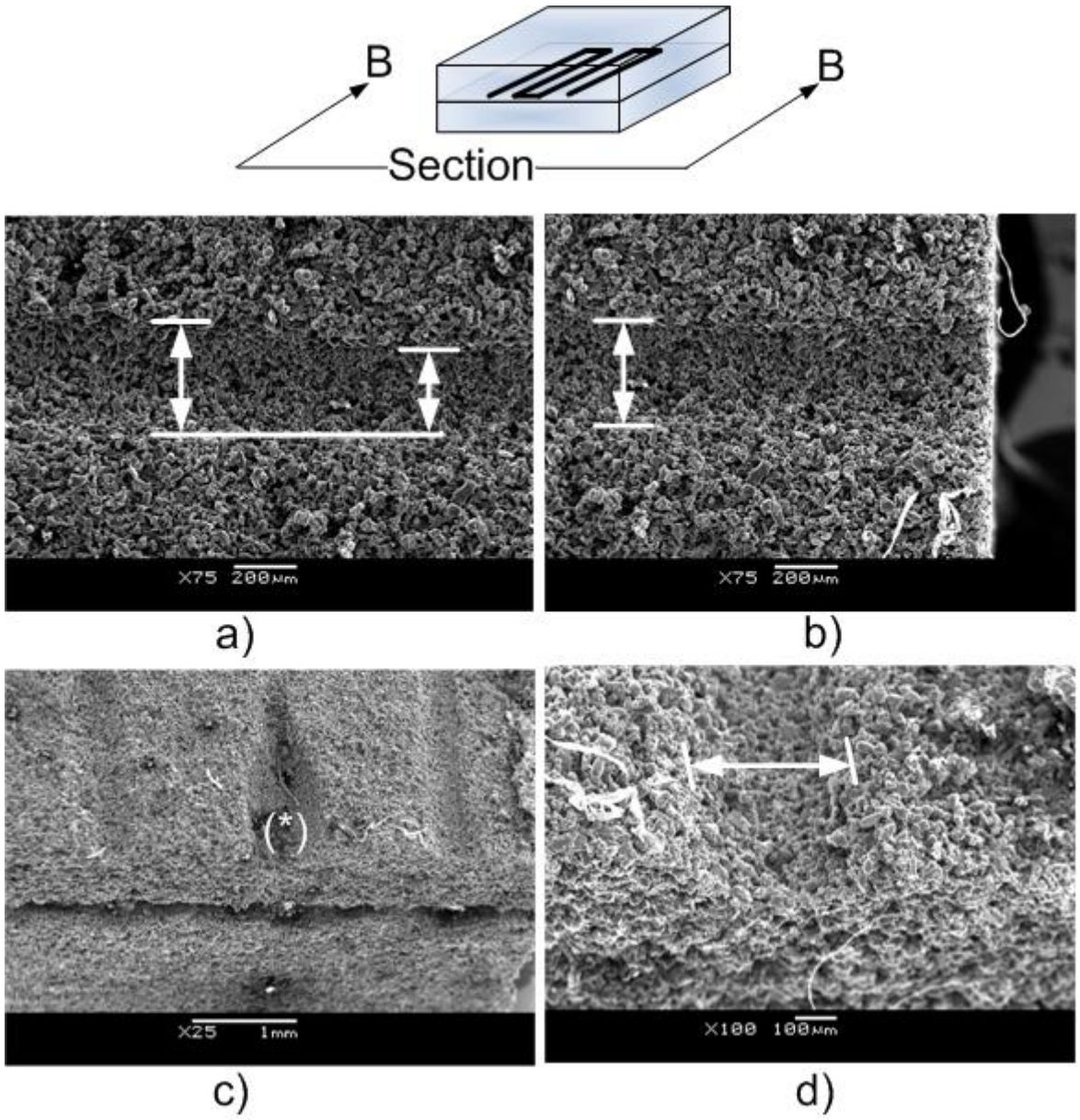

Figure 5 CPP part with integrated micro-channels after heat treatment, a) and b) top view, c) and d) view from a 30° inclination with different magnification.

3.2. Polymer Thermal Decomposition Properties

The DSC-TGA thermal analysis was performed on the EBA* and PVA respectively to determine the change in weight of the sample as a function of increasing temperature and eventually identify temperatures that are necessary to initiate thermal degradation and also the maximum temperature necessary to complete the decomposition process. The resulting DSC-TGA analysis for the photopolymer is illustrated in Figure 6a. As seen, the

decomposition becomes significant after 300˚C and it reveals one degradation region for EBA* at ~450 °C. The resulting DSC-TGA analysis for the PVA sample is illustrated in Figure 6b. As observed, the degradation process becomes significant after 200 ˚C and it peaks in two regions at 300-325 °C and 400-425 °C, where the mass loss rate is accelerated. After 500 ˚C, both photopolymer and PVA have been completely decomposed at a temperature significantly below the maximum sintering temperature of CPP (950 ˚C).

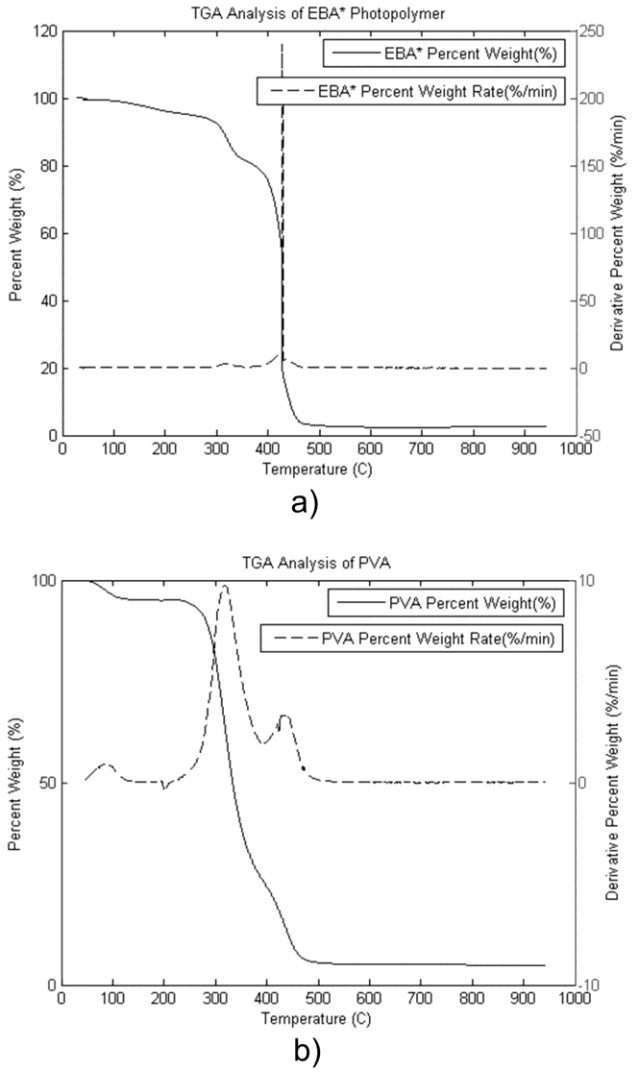

Figure 6 TGA results of (a) EBA* and (b) PVA.

3.3. Surface Chemical Composition

Figure 7 illustrates a typical result obtained through the FESEM-EDAX analysis. Table 1 summarizes the chemical composition of each sample in terms of weight percentage (wt%). The elements Calcium (Ca), Oxygen (O), Phosphorus (P), and Carbon (C) were present in both structures CPP-PVA and CPP-PVA-EBA*. Based on this analysis, it is concluded that no additional chemical elements are present in the sample produced using the proposed photopolymer. The weight percentage for both Trial 1 and Trial 2 were very similar amongst samples. The discrepancies can be attributed to the small sampling size used to determine the results.

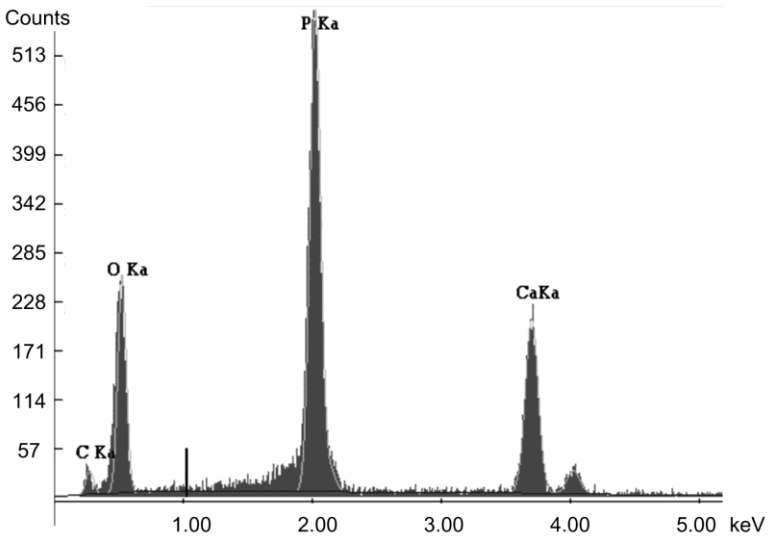

Figure 7 FESEM-EDAX analysis of a typical CPP-PVA-EBA* sample using a 250 µm^2 area

Table 1 FESEM-EDAX analysis results

Trials	Elements	CPP-PVA-EBA* (wt %)	CPP-PVA (wt %)
Trial 1 250µm^2 analysis area for each sample	C	14.63	13.94
	O	44.10	41.17
	P	26.11	27.01
	Ca	15.16	17.88
Trial 2 50µm^2 analysis area for each sample	C	14.05	15.25
	O	43.80	36.86
	P	26.56	28.37
	Ca	15.58	19.52

Figure 8 shows the XRD results indicating that there are no significant differences in peaks and intensities between the two types of samples analyzed. Based on these results, it can be inferred that there are no significant differences in chemical composition and crystallinity between the two samples and that the use of a photopolymer in the AM process does not introduce any by-products in the final product. The similarity of the XRD patterns and samples shown in our previous study [16] indicates that the resulting sintered CPP is β-CPP.

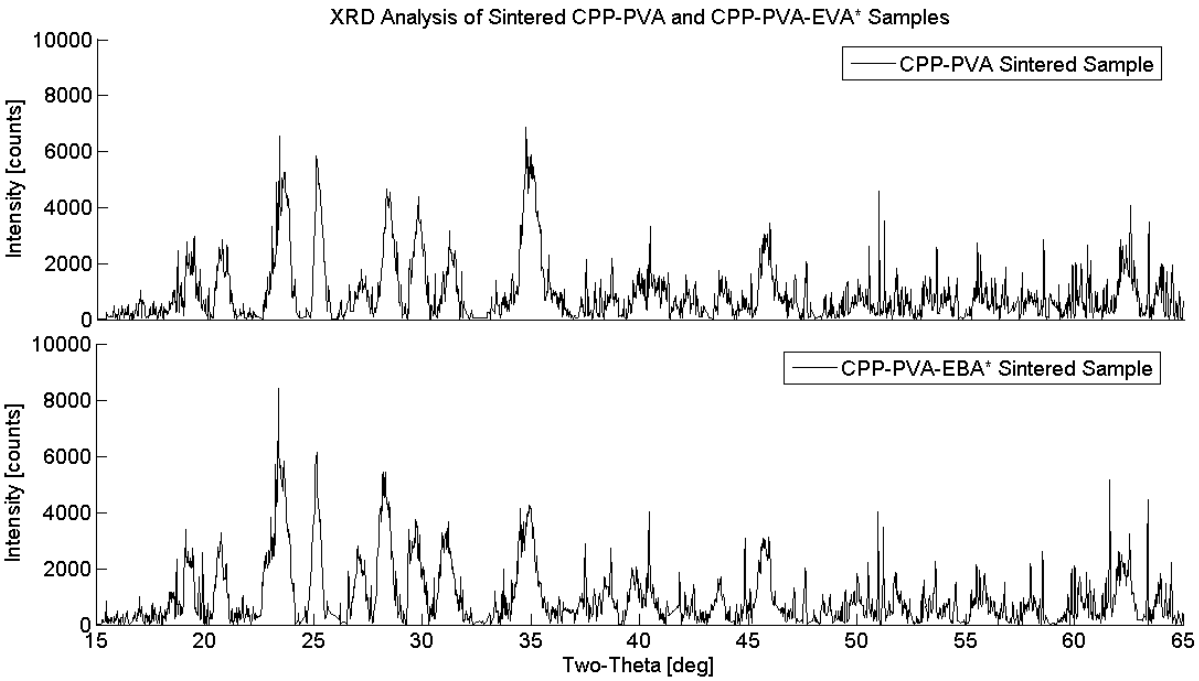

Figure 8 XRD analysis of CPP-PVA versus CPP-PVA-EBA* heat-treated samples

4. Discussion

A novel combined additive manufacturing technique was developed to achieve the formation of micro-channels within ceramic structures. This technique enables the formation of inner-channels with complex geometry and orientation. The pattern of

channels can be designed in a pre-process stage in a CAD environment. The design of the channels may be developed based upon the biological requirements of the final bio-structure and desired mechanical properties. The feature size of such channels has a direct effect on performance of the implants. Based on biological functionality, the desirable channel feature should be in the range of 100-500 μm in size [4-6]. To achieve nutrient transport and cell ingrowth, the channels must be continuous and not clogged with the constitutive material of the structure. Any attempts towards forming very small features such as complex micro-channels, pores and holes embedded in parts using the available powder-based AM processes result in clogging, since loose powder becomes trapped within the channels or pores. The developed AM-μSD technique addresses this concern, as the resulting channels within CPP structures were continuous and include small features (width of ~450μm and height of ~250μm as shown in Figure 5). The results of these feasibility studies are reflective of samples produced without applying any optimizations, which is the focus of future work.

The cross-section shape of the channels depends on the geometry of injected/deposited photopolymer tracks. The cross-sectional shape of channels may not be an important issue in bone and osteochondral tissue regeneration applications (as long as the permeability is not deteriorated) where the surface area plays an important role rather than the regularity of the channel shape. It should be noted that the post-processing stage determines the final geometry of the channels based on powder particle size and shape. The powder particle size may have a profound impact on the geometry of the channels as it affects the sintering shrinkage [17]. In addition, parts shrink during sintering when exposed to high temperature and consequently, the geometrical feature of the channels change as CPP particles start to form sinter-necks and may rearrange after photopolymer burn-off. Consequently, the

sintering protocol may affect the size and shape of the features. Furthermore, CPP structures made via AM-μSD are likely to shrink anisotropicly if the shape of the particles has an aspect ratio larger than one [14]. The different shrinkage in the directions parallel and perpendicular to constitutive layers may contribute to the irregular shape of the channels.

In addition, the deposition of the photopolymer tracks on the powder substrate is affected by the flatness of underlying powder layer spread by the counter rotating roller which, in turn, is drastically affected by the particle size. The finer the particle size is, the smoother the powder bed will be, which results in a more uniform photopolymer deposition.

The channels were originally spaced 2 mm apart, however, due to part shrinkage during the post-processing heat treatment, the distance between the channels was reduced by ~12.5%. This value should be further investigated and taken into account as a compensation factor in the CAD design stage. A future study will focus on optimizing the size and integrity of the channels by tuning the μSD injection parameters to reduce the feature size and the UV light exposure time and height to optimize the shape integrity.

Material characterization studies including TGA and XRD analyses also revealed that there was no residual by-product left from the photopolymer after the heat treatment stage. This matter is very crucial for evaluation of the developed method since any residual by-product material in the final part may adversely affect the biological properties. A comparison between the TGA analysis for the two polymers shown in Figure 6 suggests that both followed the same degradation trend; however, the degradation of PVA occurred over a higher temperature range (200 to 450 °C) while EBA* was almost entirely degraded around 450°C. In both cases, the equilibrium weight does not stabilize about zero which may be due to the system errors associated with initial calibration, as no visible residue was found

after the heating sequence [18]. However, the degradation curves stabilize significantly below the highest curing temperature of 950 °C, which is the topic of interest for this experiment analysis. In addition, XRD patterns of the CPP-PVA-EBA* and CPP-PVA samples confirm that the sacrificial photopolymer does not influence the crystalline structure of the post-processed part. It can be concluded that the material system was selected properly for the purposes of forming channels within bio-structures.

In the present study, the formation of micro-channels in planes parallel to the direction of layers was examined. The formation of channels with complex shapes and/or conformal configuration will be investigated in future work.

4. Conclusions

This study introduced a novel combined additive manufacturing and micro-syringe deposition (AM-μSD) process for manufacturing bio-ceramic structures with micro-size channels for bone and osteochondral tissue regeneration. This methodology addresses current obstacles associated with powder-based additive manufacturing in terms of embedding complex channels with a small feature size within the internal architecture of parts. In this work, it was shown that these obstacles can be addressed using the newly developed AM-μSD protocol by producing porous ceramic structures with embedded internal channels with features in the range of ~200-500 μm which are desirable for bone repair. A material system was proposed for this manufacturing technology to fulfill an important material design criterion, which is the prevention of any harmful residuals in the final product after the heat treatment protocol. The experimental analysis showed no signs of pyrolysis residuals left in the CPP structures, and no additional phase was observed in crystalline CPP material. As a result, the injectable photopolymer can effectively be used

in a combined AM-μSD process to act as a sacrificial element required for making complex micro-scale channels inside biodegradable ceramic structures.

5. Acknowledgments

This work was funded by the Department of Defense US Army (Medical Research Acquisition Activity- Grant number of OR090169).

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List of Figures

Figure 1 Natural bone morphology and a potential network of channels and/or macro-pores that can be integrated in the implant structure to mimic natural bone architecture.

Figure 2 Schematic of the novel AM-μSD fabrication process for production of entire implants in a layer-by-layer fashion through sequential Steps 1 to 4.

Figure 3 a) and b) views of in-house developed AM-μSD fabrication system, c) close-up view of a typical manufactured layer presenting the deposited photopolymer structure on the powder substrate.

Figure 4 Cross section of CPP part with integrated channels after heat treatment.

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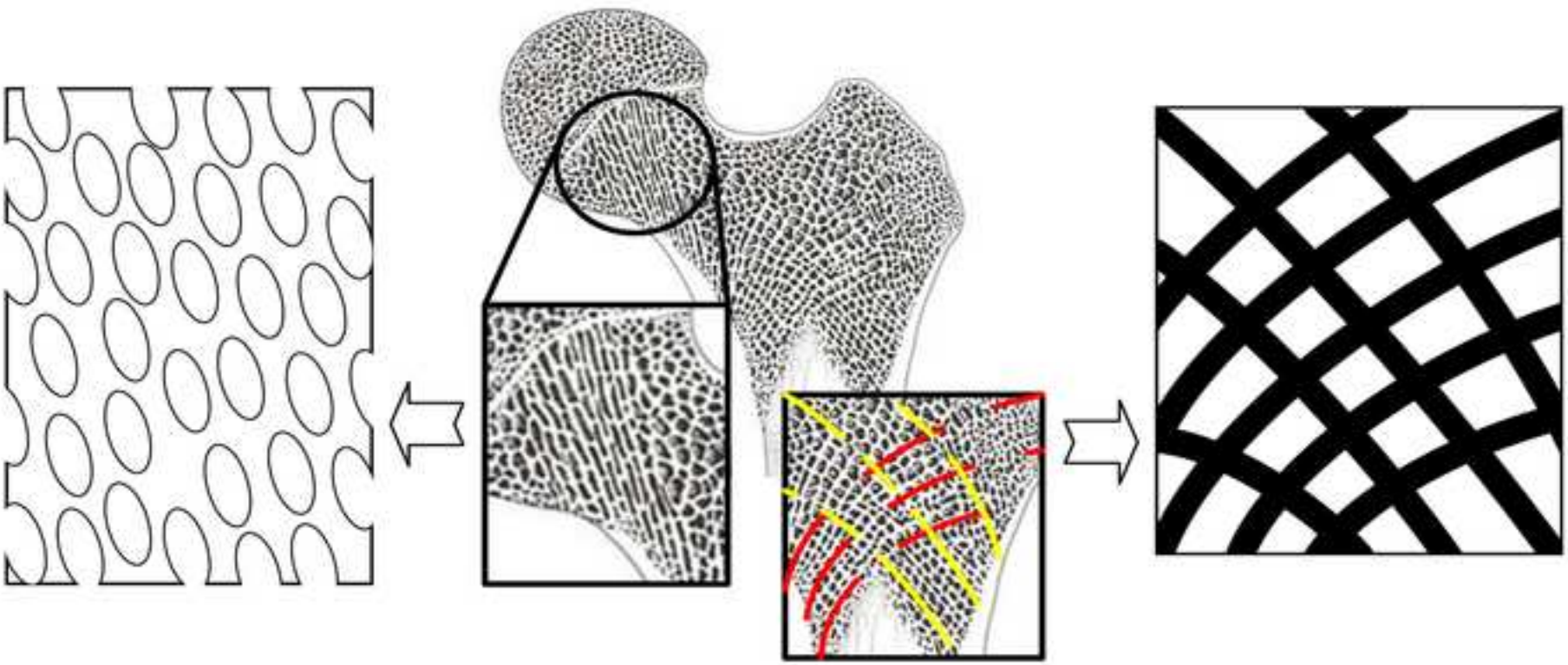
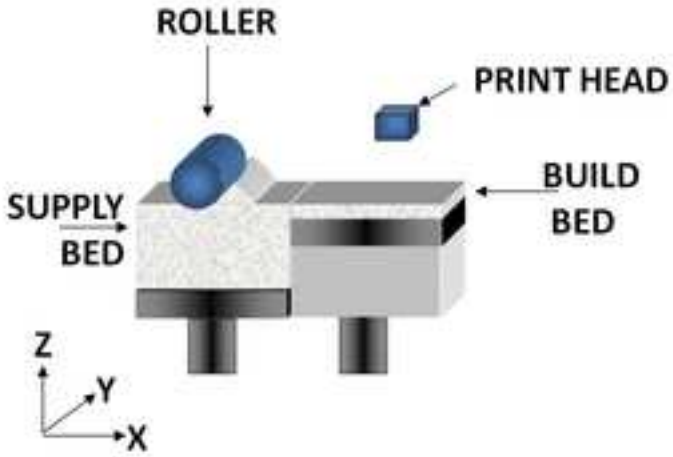
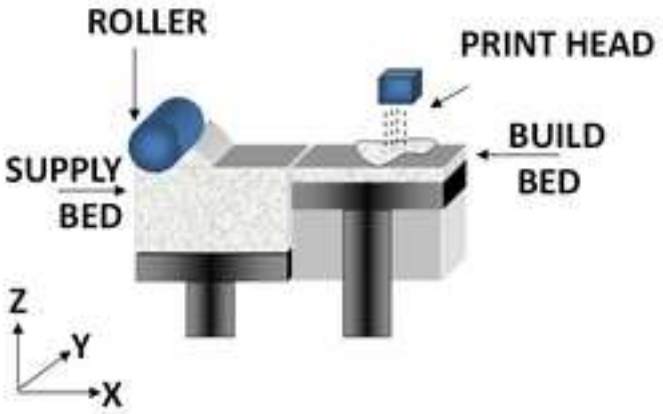
List of Tables

Table 1 FESEM-EDAX analysis results

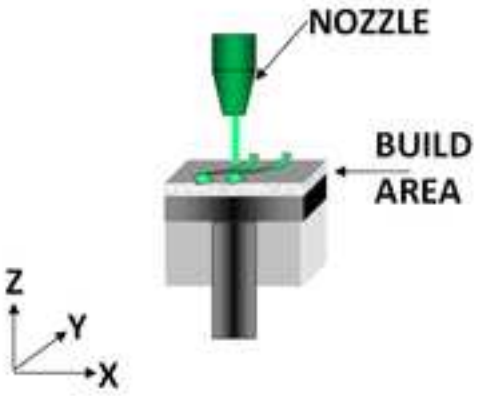




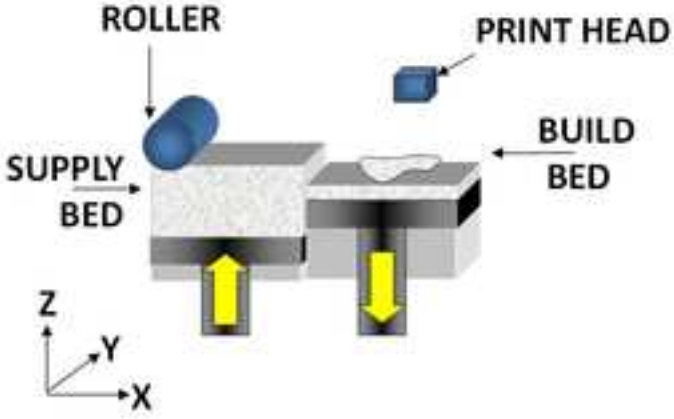
Step1:
Spread new powder layer using a custom roller mechanism.



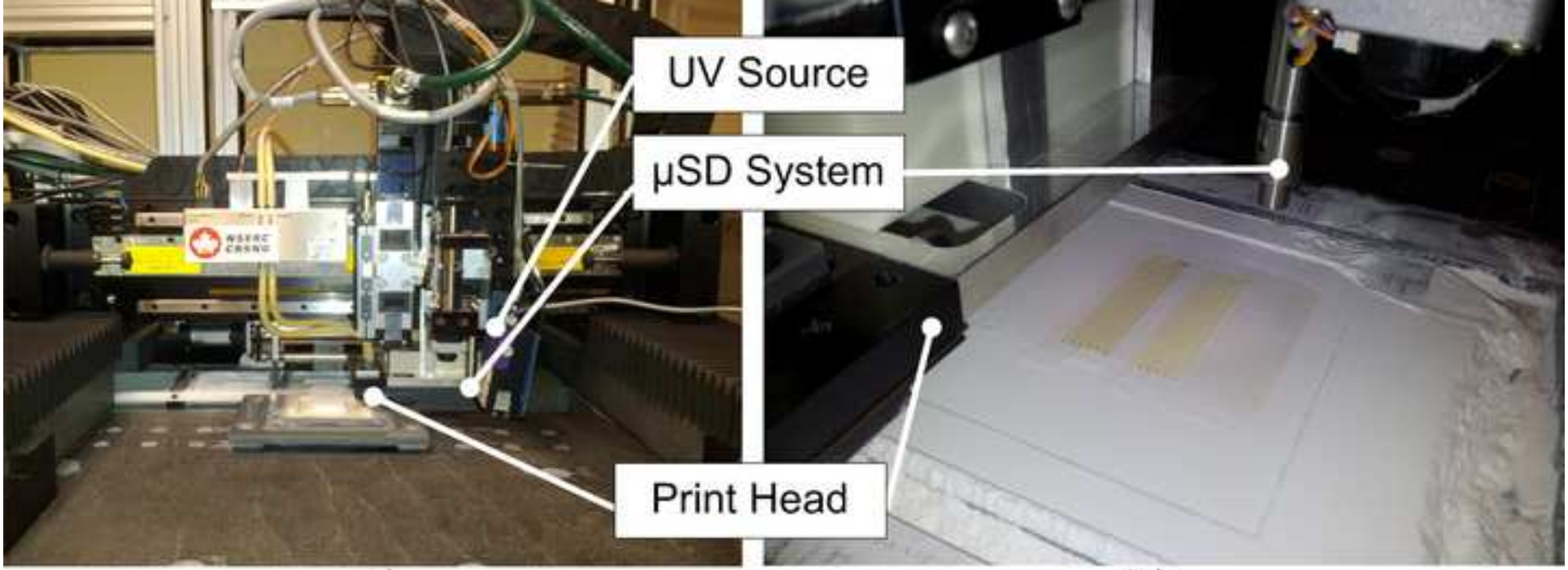
Step 2:
Inject binder using print-head based on CAD layer data



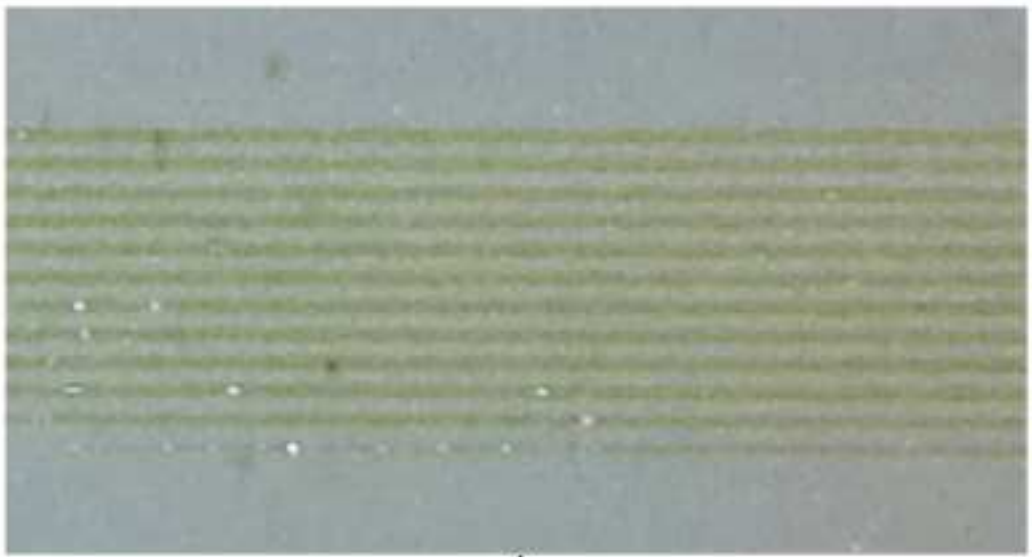
Step 3:
Extrude photopolymer through a nozzle, based on layer specs



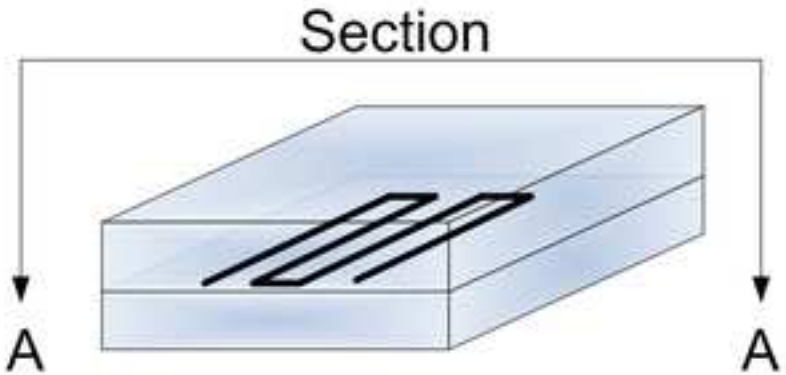
Step 4:
Lower build bed, raise supply bed, deposit new powder layer



a) b)



c)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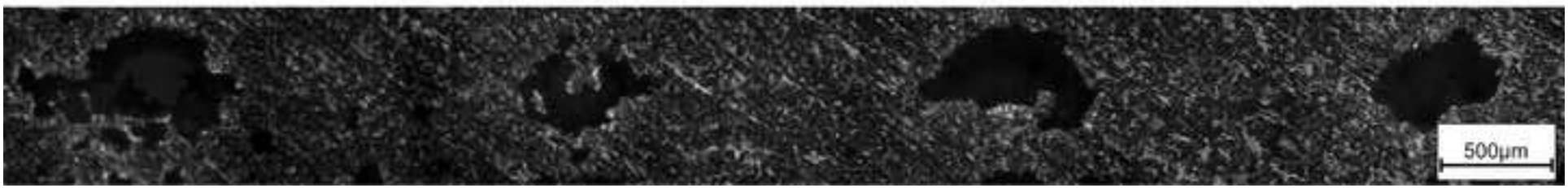
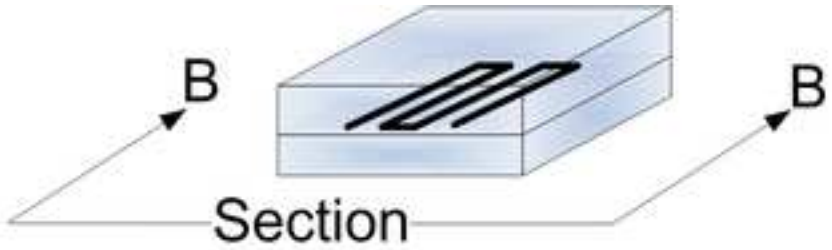
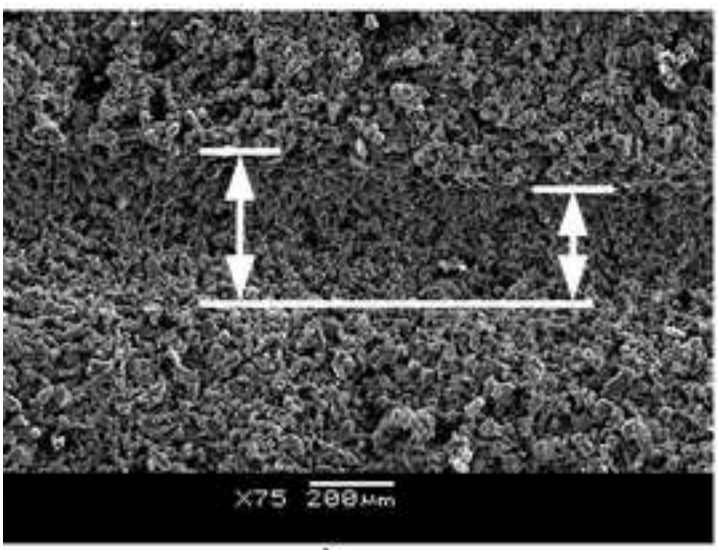
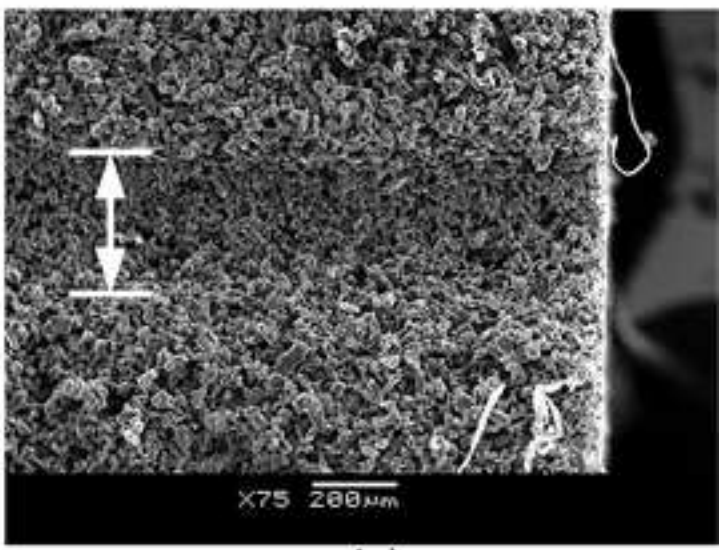

500µm

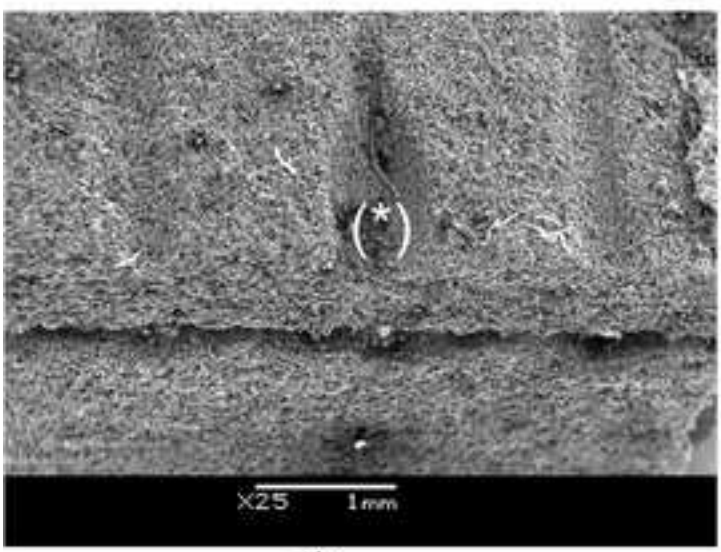




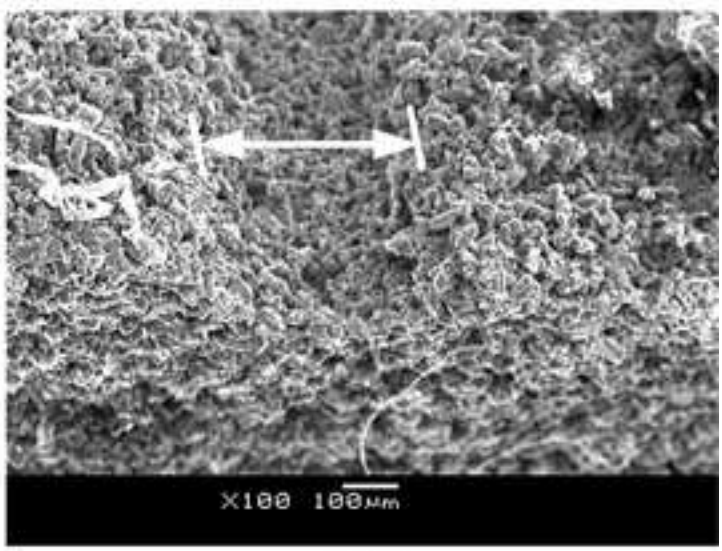
a)



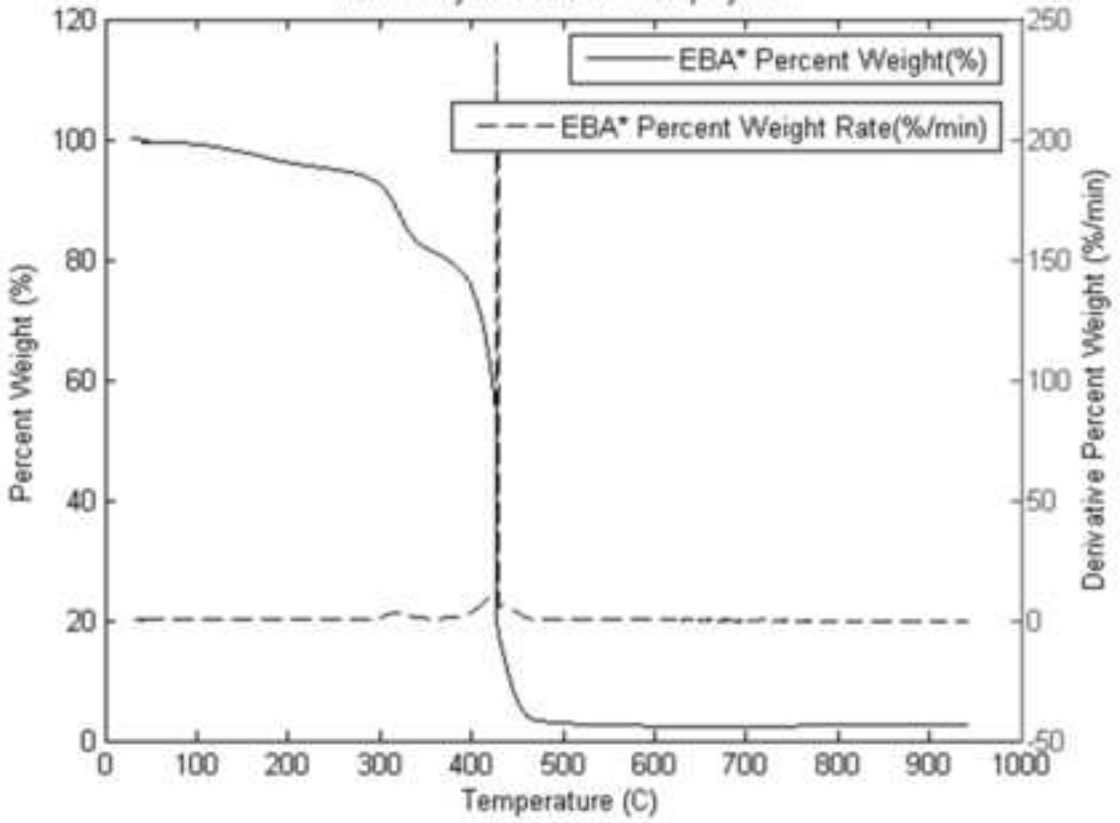
b)



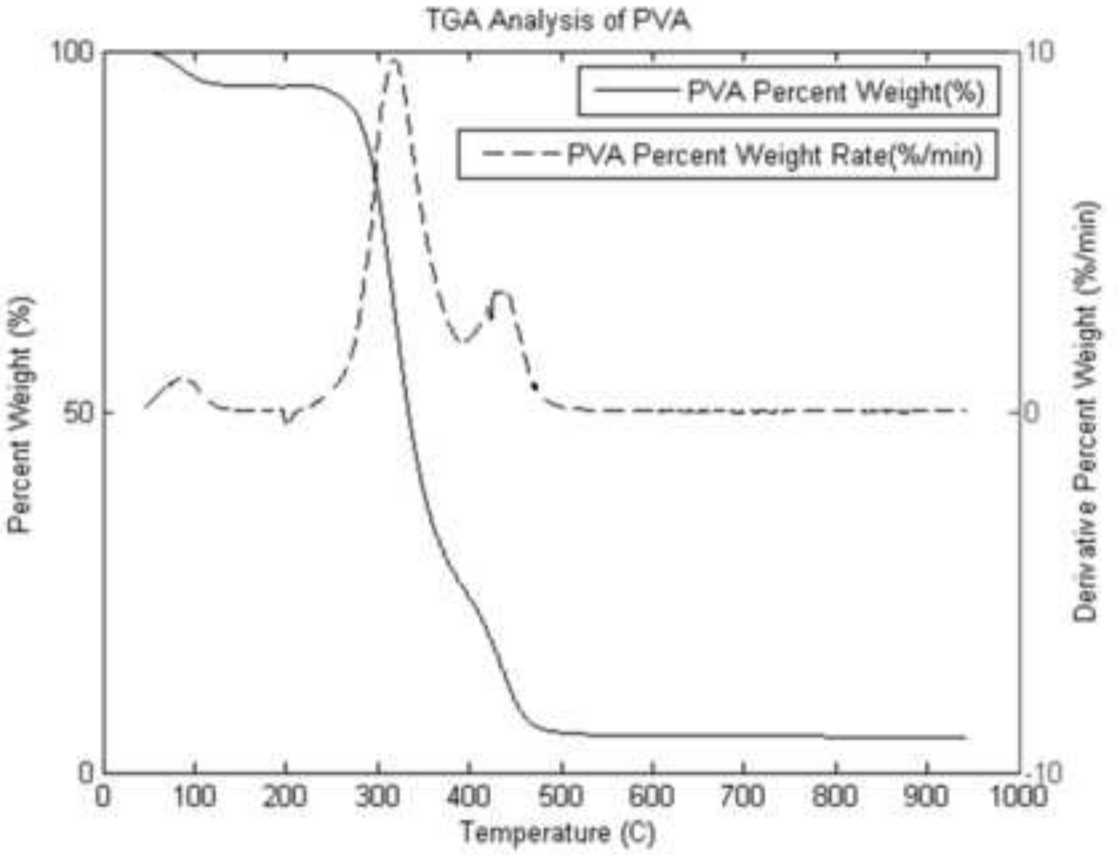
c)



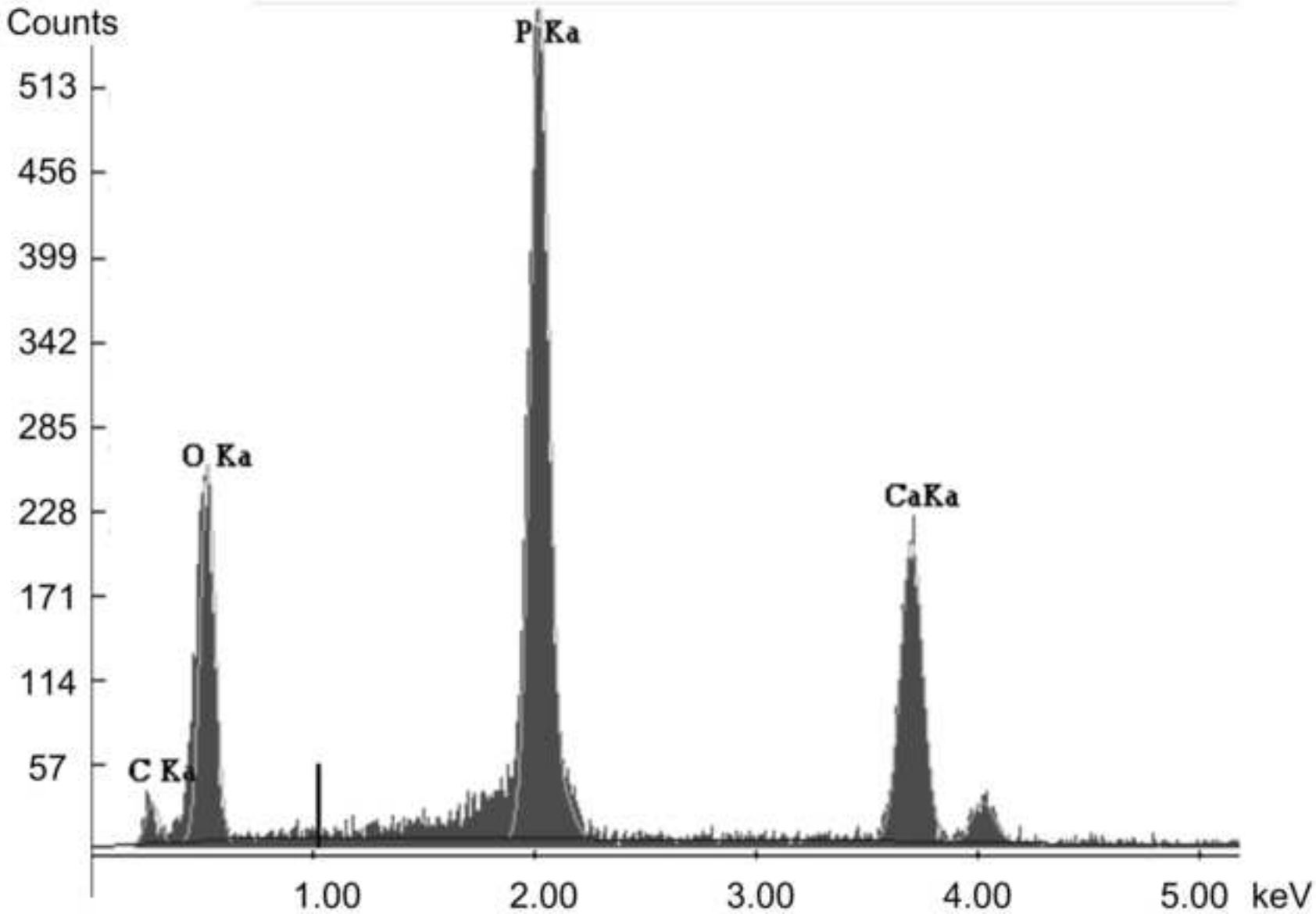
d)


EBA* Percent Weight(%)
EBA* Percent Weight Rate(%/min)
Percent Weight (%)
Derivative Percent Weight (%/min)
Temperature (C)
0
20
40
60
80
100
120
-50
0
50
100
150
200
250
0
100
200
300
400
500
600
700
800
900
1000

a)


TGA Analysis of PVA
PVA Percent Weight(%)
PVA Percent Weight Rate(%/min)
Percent Weight (%)
Derivative Percent Weight (%/min)
Temperature (C)
0
50
100
-10
0
10
0
100
200
300
400
500
600
700
800
900
1000

b)


Counts
513
456
399
342
285
228
171
114
57
P Ka
O Ka
CaKa
C Ka
1.00
2.00
3.00
4.00
5.00
keV

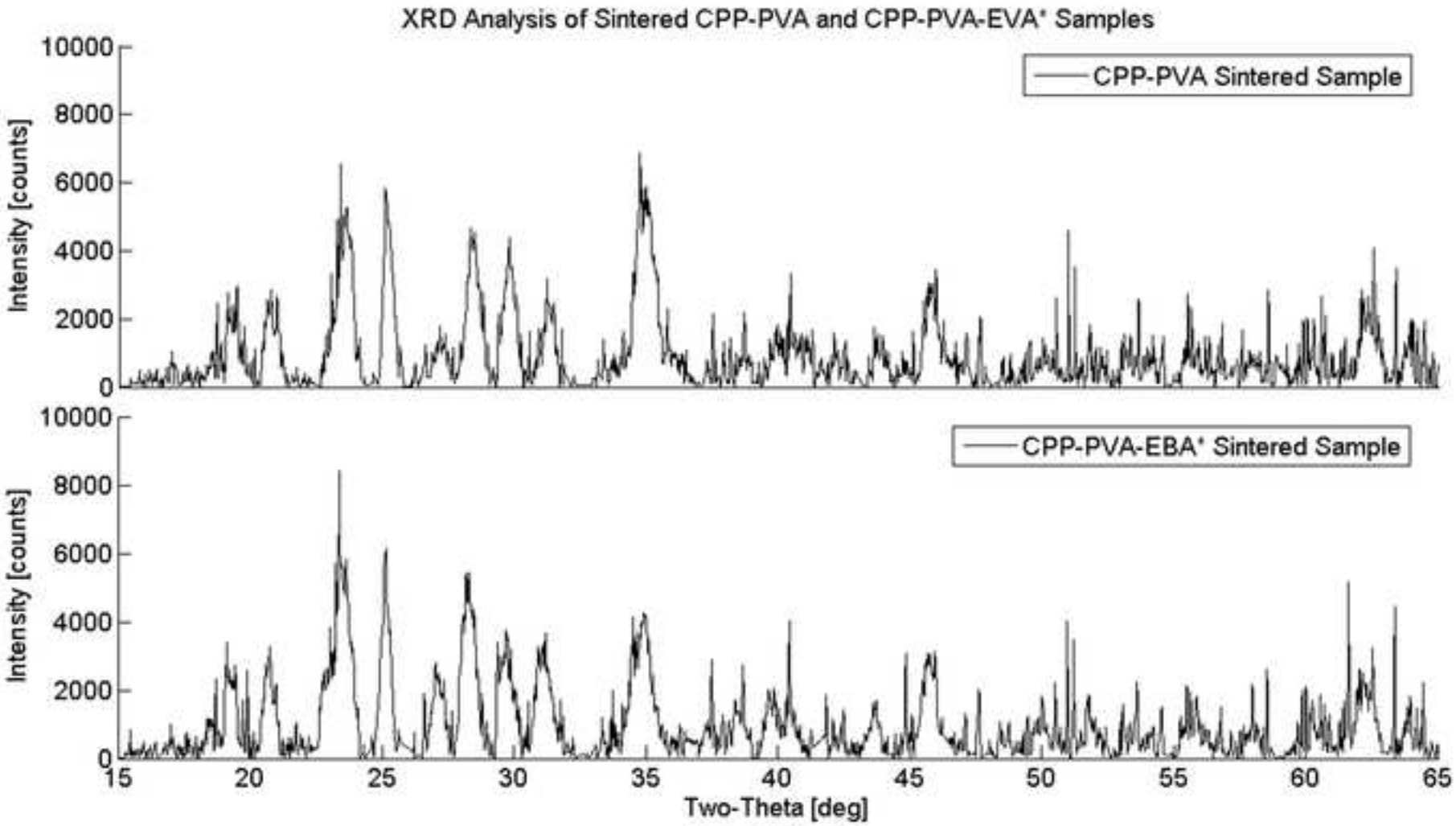

XRD Analysis of Sintered CPP-PVA and CPP-PVA-EVA* Samples
CPP-PVA Sintered Sample
CPP-PVA-EBA* Sintered Sample
Intensity [counts]
Two-Theta [deg]
0
2000
4000
6000
8000
10000
15
20
25
30
35
40
45
50
55
60
65

Table 1 FESEM-EDAX analysis results

Trials	Elements	CPP-PVA-EBA* (wt %)	CPP-PVA (wt %)
Trial 1 250μm^2 analysis area for each sample	C	14.63	13.94
	O	44.10	41.17
	P	26.11	27.01
	Ca	15.16	17.88
Trial 2 50μm^2 analysis area for each sample	C	14.05	15.25
	O	43.80	36.86
	P	26.56	28.37
	Ca	15.58	19.52

Characterization of Calcium Polyphosphate Scaffolds with Embedded Micro-Channels for Osteochondral Tissue Replacement or Augmentation

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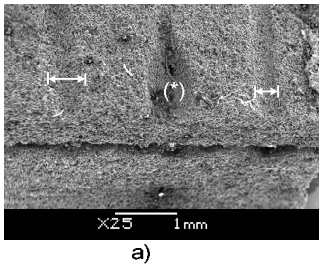
[1]University of Waterloo, Waterloo, ON, Canada, [2]Mount Sinai Hospital, Toronto, ON, Canada

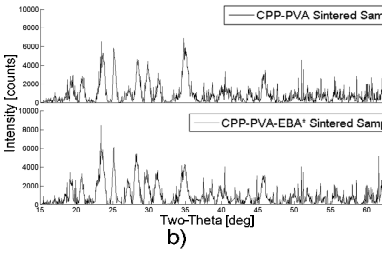
Introduction: This study addresses the design, manufacturing and characterization of bio-ceramic scaffolds with complex networks of interconnected micro-channels for osteochondral tissue replacement and augmentation. For such scaffolds, it is hypothesized that organized networks of micro-channels encourage bone in-growth through promoting metabolic exchange and vascularization [1, 2]. The proposed bio-ceramic implants were manufactured using a powder-based layer-by-layer Additive Manufacturing combined with a Micro-Syringe Deposition (AM-μSD) platform, where sacrificial photopolymer patterns were injected within the required bio-ceramic powder layer. This novel process requires a heat treatment step to sinter the scaffolds and to remove the sacrificial element to form micro-scale networks of channels within. The study focuses on the geometrical characterization of internal architecture of implants to quantify the performance of the manufacturing platform, mechanical analysis of implants to ensure adequate strength for osteochondral replacement or augmentation, material characterization to demonstrate that there is no cytotoxic pyrolysis residue post sintering, and in vitro biological study to quantify cell viability and ingrowth within the proposed implant.

Materials and Methods: The photopolymer solution is 56%wt ethoxylated(10 bisphenolAdiacrylate) monomer, 13%wt cellulose acetate butyrate, 0.5%wt phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide dissolved in 30.5%wt acetone. Calcium polyphosphate (CPP) powder (<45μm particle size) was used as scaffold material with polyvinyl alcohol 7wt% solution in de-ionized water as a binder. Structural and geometrical characterization was performed using scanning electron microscopy. Porosity was measured using mercury intrusion porosimetry. Mechanical characterization in uniaxial compression was performed via 1-kN load-cell. The thermal degradation profile of the photopolymer was analyzed via Differential Scanning Calorimetry Thermo Gravimetric Analysis (DSC-TGA). X-Ray Diffraction (XRD) was used to examine the material and crystalline composition of the heat-treated implant. In vitro cell culturing was performed using MG-63 cell line osteosarcoma (ATCC, CLR-1427, US) in Minimum Essential Medium Eagle (ATCC, 30-2003, US) base medium for 3-5 weeks culture periods.

Results and Discussion: Channels with a feature size of 225-300μm were produced within 35% porous structures, as illustrated in Figure 1a. These future sizes are suitable for cell ingrowth and transport of nutrition and waste material [1, 2]. The mechanical characteristics were shown to be within the acceptable range for osteochondral tissue engineering, with a compressive strength within a range of 30-50MPa, comparable with the strength of samples without channels [3]. The chemical and crystalline characterization shown in Figure 1b illustrates that the there was no significant pyrolysis residue in the developed part and that the resulting sintered part was β-CPP, a material with proven biocompatibility [4]. *In vitro* cell culture tests showed that the scaffold internal network channels promoted cell migration, attachment, proliferation and penetration. An analytical study is underway to compare the biological performance of scaffolds with and without internal channels.



a)



b)

Figure 1 a) CPP part with integrated channels after heat treatment viewed under SEM (*defect from dissecting).
Figure 1 b) XRD analysis of heat-treated samples with and without channels. There are no significant differences in peaks and intensities between the two types of samples analyzed.

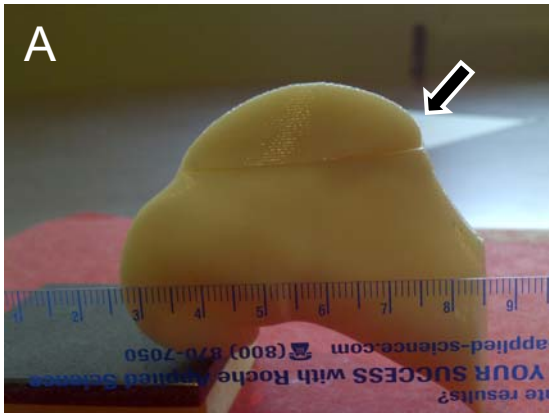
Conclusions: This study proposed a novel manufacturing method for fabrication of bio-ceramic implants with micro-size channels for bone and osteochondral tissue regeneration. The results showed that these types of implants and the manufacturing platform can be an appropriate pathway for osteochondral tissue engineering from a mechanical, chemical and biological perspective.

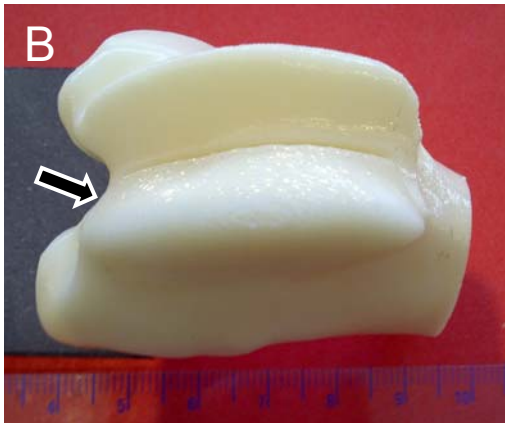
References [1] Martin I. J Biomech 2007;40:750-65. [2] Kandel RA. Biomaterials 2006;27:4120-31.
[3] Shanjani Y. Acta Biomater 2011;7:1788-96. [4] Grynpas M. Biomaterials 2002;23:2063-70.

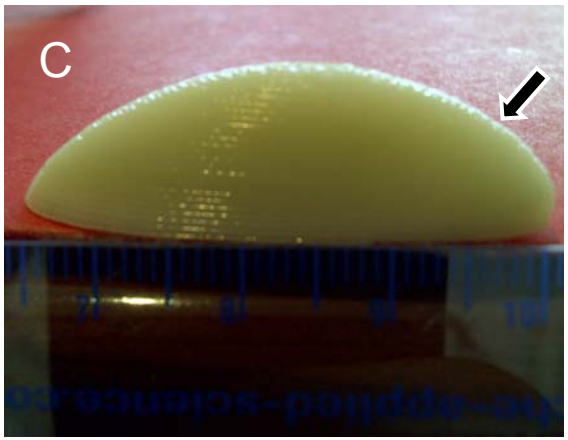
SUPPORTING DATA

Figure 1A

FEMORAL CONDYLE SEGMENT MODEL



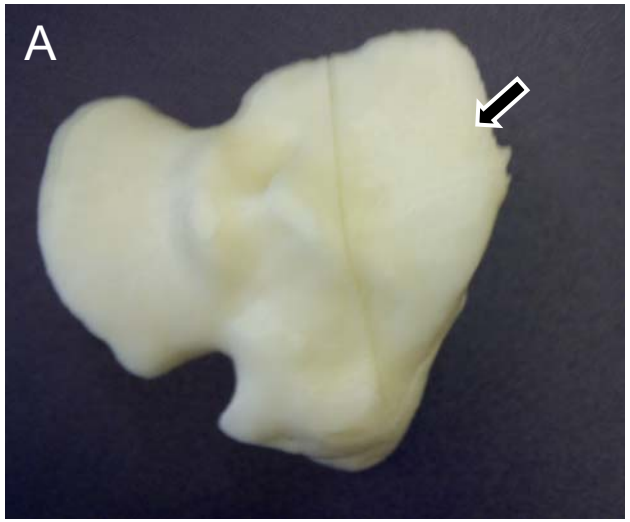


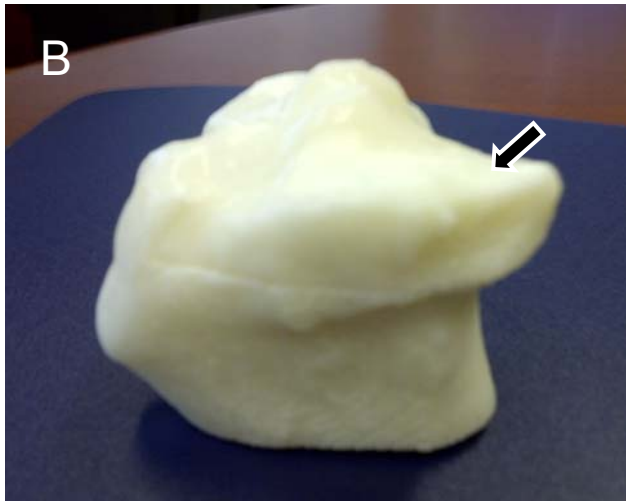


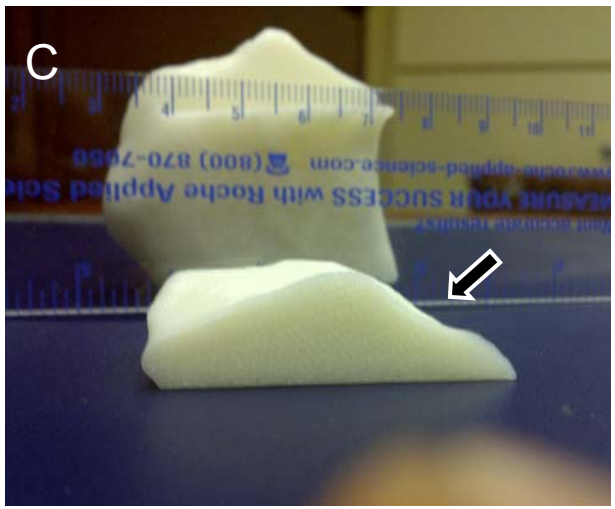
Shaped femoral condyle segment model (arrow) generated by solid freeform fabrication and placed in a model of a sheep condyle as viewed from side (A) and top (B). The implant alone as seen from the side (C).

Figure 1B

MEDIAL TIBIAL PLATEAU MODEL

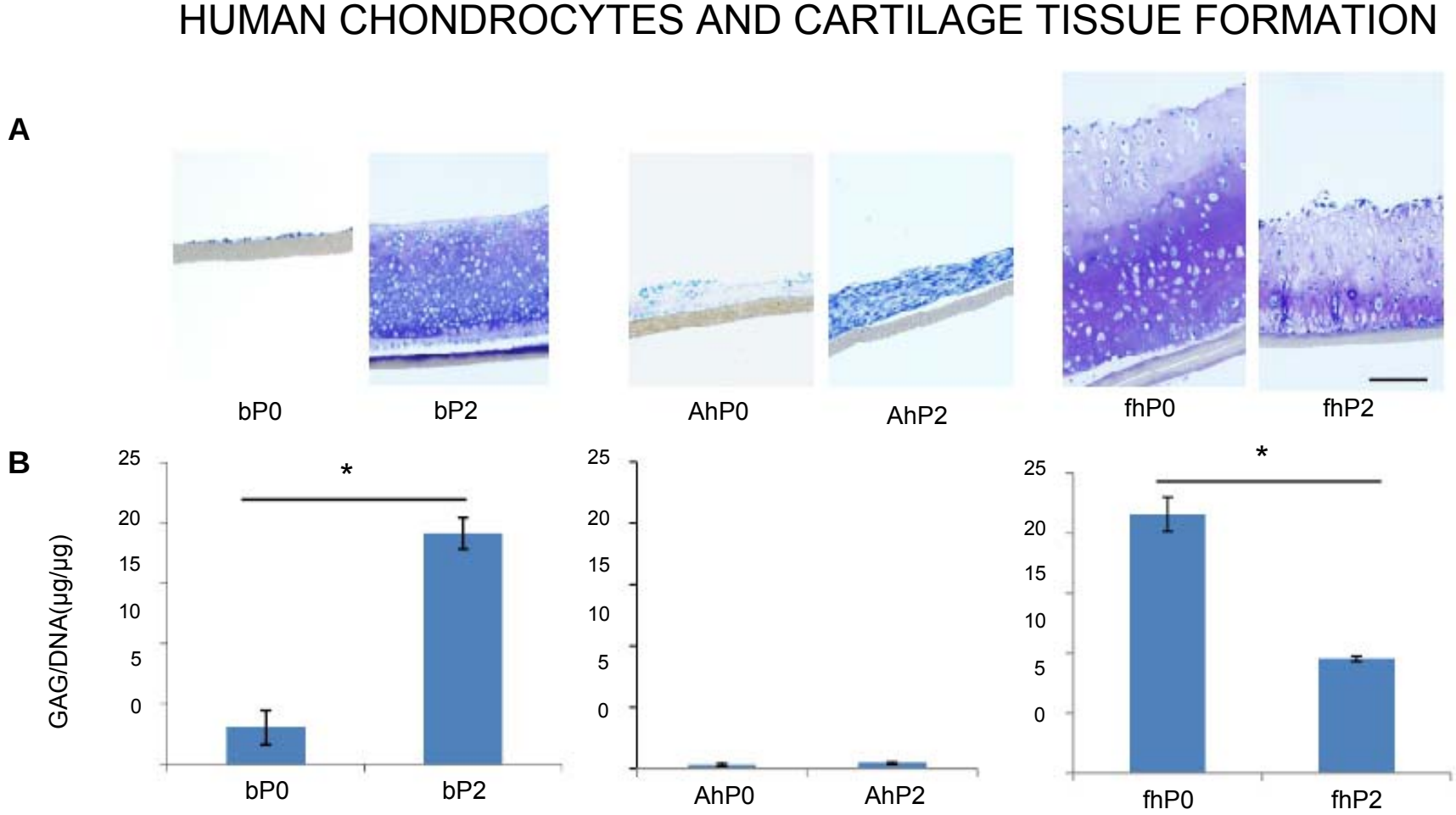






Shaped medial tibial plateau model (arrow) generated by solid freeform fabrication and placed in a sheep tibial plateau model as viewed from top (A) and side (B). The implant alone as seen from the side (C).

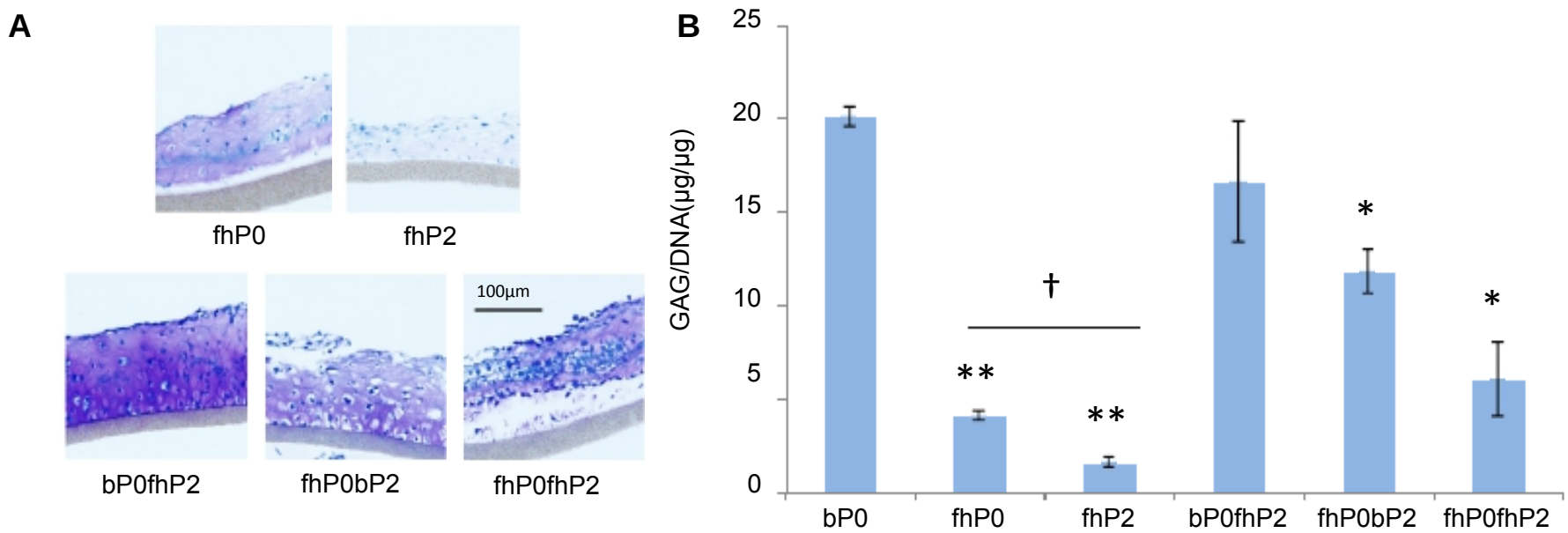
Figure 2



(A) Photomicrographs of histological sections showing that fetal human chondrocytes form cartilage tissue by 3 weeks in culture (fhP0) in contrast to adult chondrocytes (AhP0 and AhP2). Passaged fetal chondrocytes do not form hyaline cartilage (fhP2). As a control, the cartilage tissue formed by passaged bovine chondrocytes (hP2) is shown. Primary bovine chondrocytes (bP0) did not form cartilage in vitro. (B) Quantification of proteoglycan content in these tissues. *Statistically significant $p<0.05$.

Figure 3

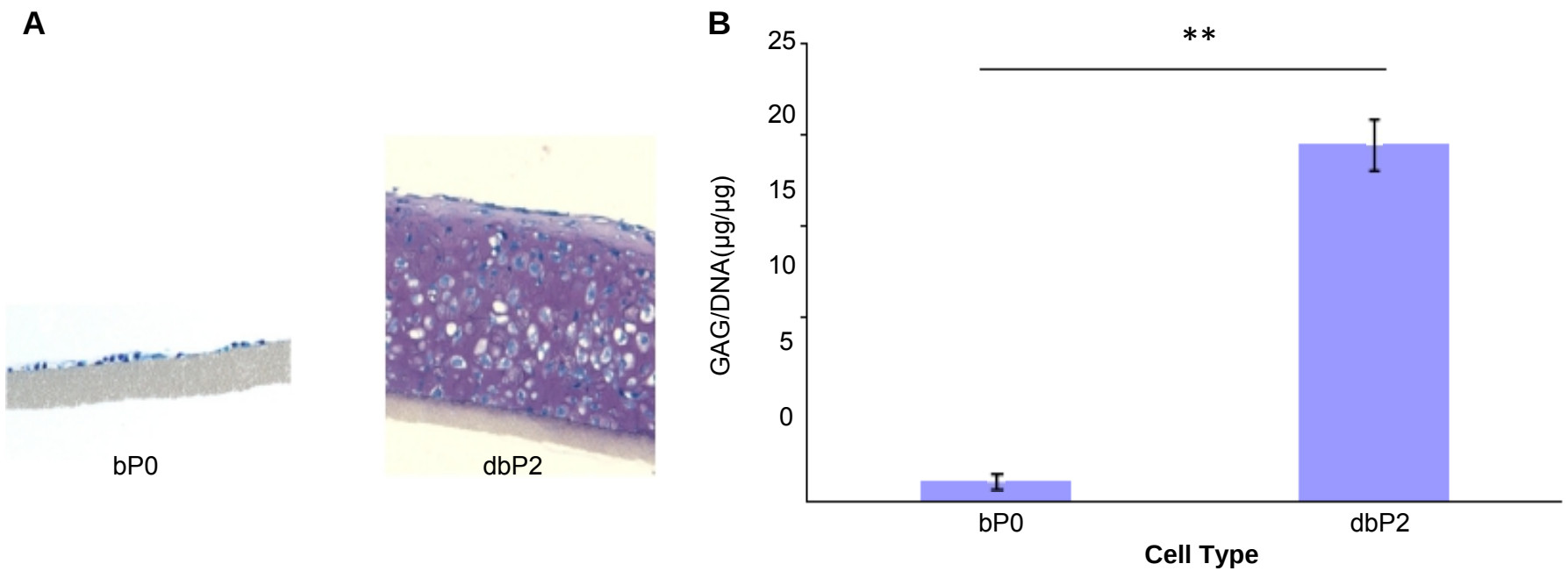
HUMAN CHONDROCYTE CO-CULTURE STUDIES



(A) Bovine chondrocytes induce passaged fetal chondrocytes by 3 weeks in culture to form cartilage tissue (bP0fhP2) when grown in side-by-side co-culture. In contrast, co-culture of fetal chondrocytes, primary chondrocytes with passaged chondrocytes (fhP0fhP2) did not result in cartilage tissue formation by the passaged cells. Fetal chondrocytes could not induce hyaline cartilage tissue formation by passaged bovine chondrocytes (fhP0bP2). (B) Quantification of proteoglycan content in the in vitro tissues formed by passaged chondrocytes. Statistically significant to bP0, *$p<0.05$; **$p<0.01$; † Statistically significant $p<0.05$.

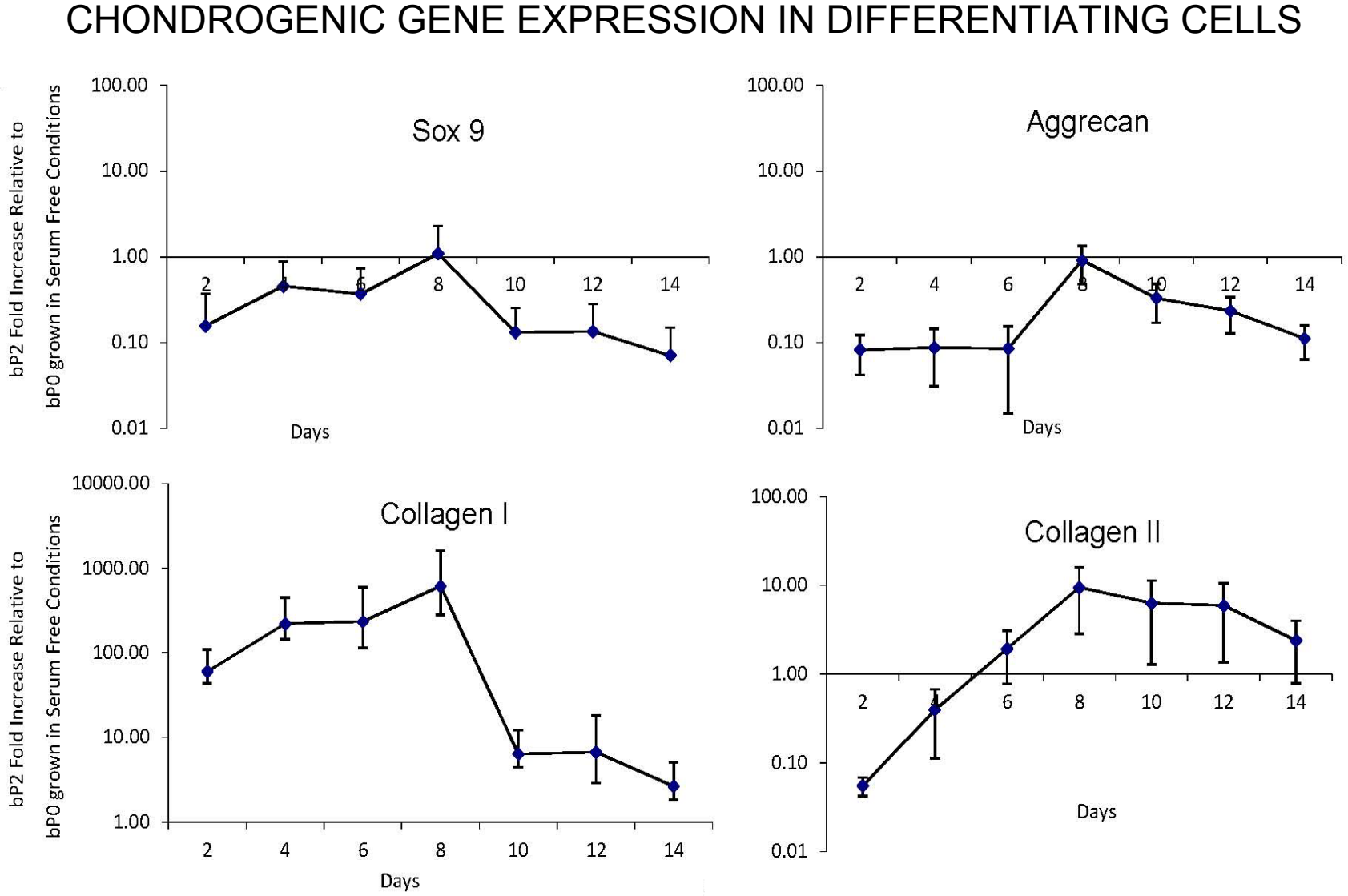
Figure 4

CHONDROCYTES GROWN SERUM-FREE FORM CARTILAGE



Bovine chondrocytes grown under serum-free conditions form hyaline cartilage tissue as seen histologically (A) and confirmed biochemically (B). **p<0.01.

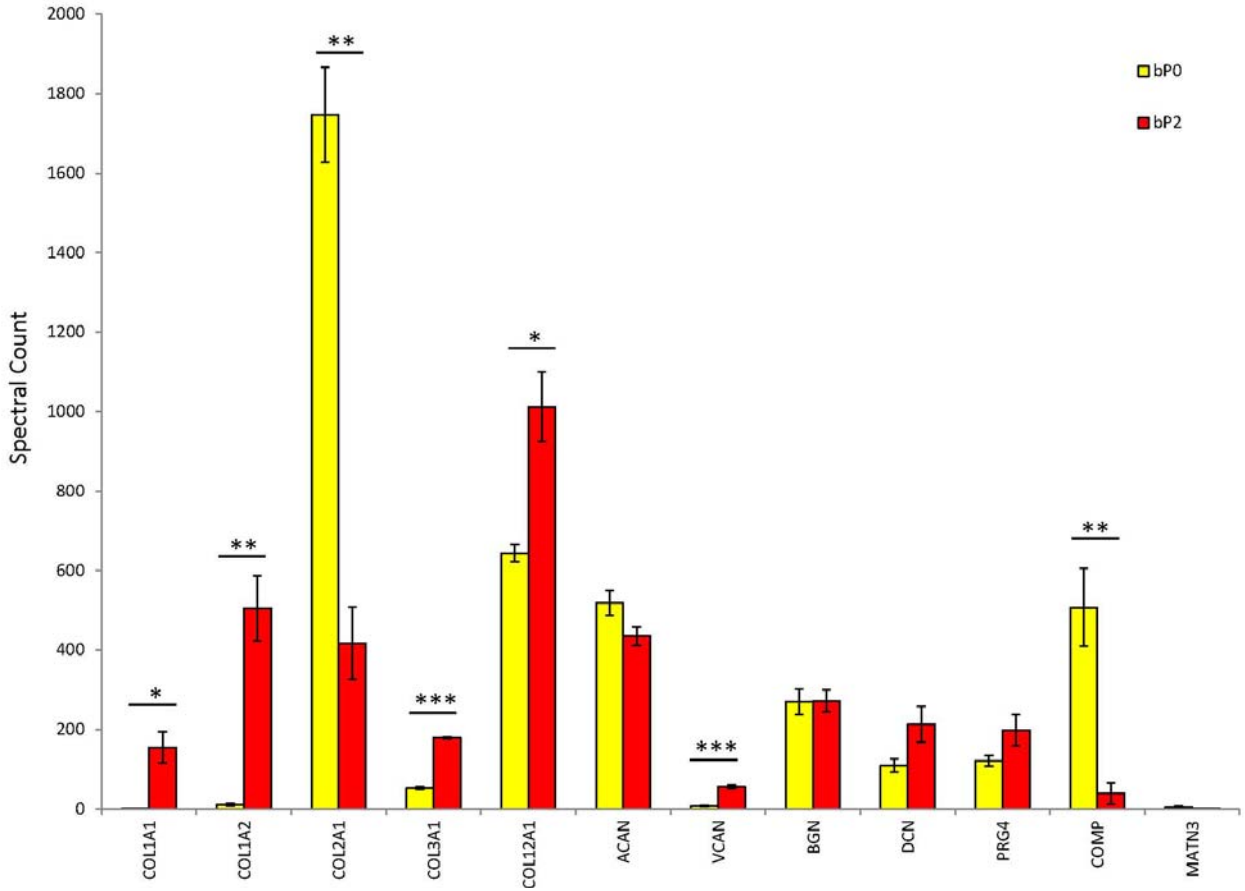
Figure 5

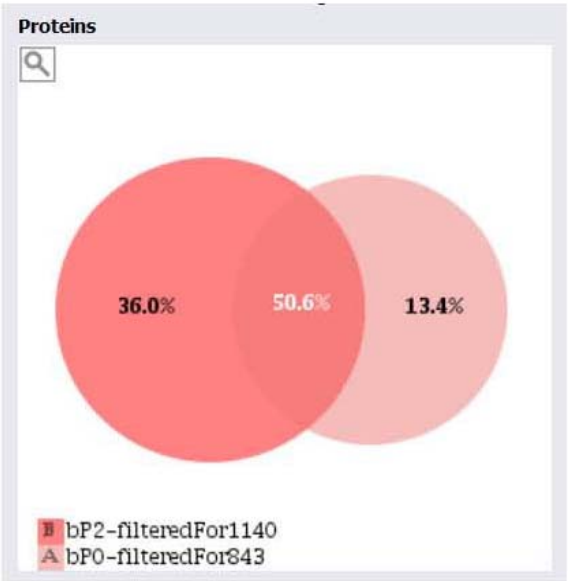


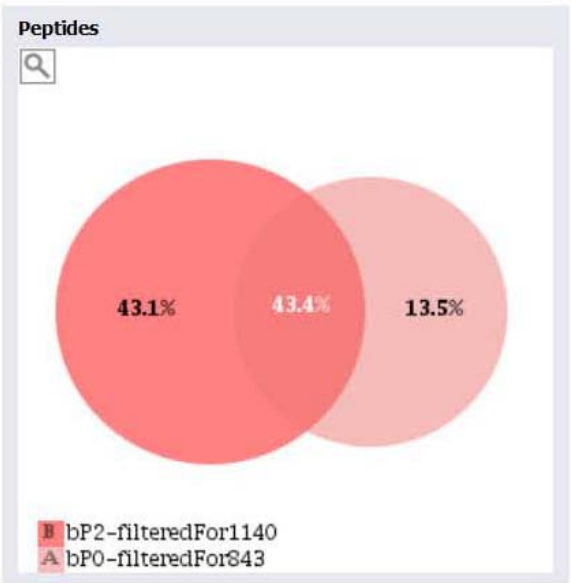
Chondrogenic gene expression profiles over time in redifferentiating cells over the first 14 days of culture.

Figure 6

MASS SPECTROSCOPIC ANALYSIS OF CONDITIONED CULTURE MEDIA







Proteonomic analysis of secretome of differentiating cells compared to primary chondrocytes that do not form cartilage. *p<0.05; **p<0.01; ***p<0.001.